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DISPOSITION AND METABOLISM OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE IN FEMALE FISCHER 344 RATS AND B6C3F$_1$ MICE

by

Kevin Lyndon Salyers

A Dissertation Submitted to the Faculty of the

COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)

In Partial fulfillment of the Requirements
For the Degree of

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In the Graduate College

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Kevin Lyndon Salyers

entitled DISPOSITION AND METABOLISM OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE IN FEMALE FISCHER 344 RATS AND B6C3F$_1$ MICE.

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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I dedicate this dissertation to my wife Reneé whose unconditional support, encouragement, and unselfish love made it all possible.

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ABSTRACT

4-Vinyl-1-cyclohexene diepoxide (VCD) is a known carcinogen, a direct acting mutagen, and an ovarian toxicant. Female B6C3F$_1$ mice are more susceptible to the ovarian toxicity of VCD than female Fischer 344 rats, a difference which may be explained by the disposition/metabolism of VCD. Following a single dose ^{14}C-VCD was rapidly metabolized and excreted predominantly in the urine of both species. However, the excretion rate was higher in the female rats compared to female mice. The major metabolite present in rat urine was the 4-(1,2-dihydroxy) ethyl-1,2-dihydroxycyclohexane (tetrol). However, in the mouse, the tetrol was only a minor metabolite. The major metabolites appeared to be polar conjugates of VCD, which also appeared in rat urine. This difference in urinary metabolites indicates different metabolic pathways of VCD between the two species. The rat hydrolyzes VCD by epoxide hydrolase to form the tetrol and also conjugates VCD. The mouse conjugates VCD but has a reduced capacity to hydrolyze the epoxide moieties.

To further characterize the disposition of VCD, blood kinetic parameters were investigated. Although both species rapidly cleared VCD from the plasma, the mouse had a shorter plasma $t_{1/2}$ and mean residence time (MRT). The volume of distribution was also five fold greater in the mouse compared to the rat. Therefore, VCD distributes from the plasma to tissues, possibly target tissues (ie. ovaries), more profoundly in the mouse than in the rat.

In vitro metabolism studies were performed to determine if differences in epoxide hydrolase explain the inability of the mouse to excrete the tetrol metabolites. Although the K_m was similar, the V_{max} for conversion of VCD to the tetrol was four-fold higher in rat

hepatic microsomes compared to those of the mouse. Therefore, differences in the disposition and metabolism of VCD exist between female Fischer 344 rats and $B6C3F_1$ mice. These differences explain, in part, the increased susceptibility of the mouse ovary to the toxic effects of VCD.

CHAPTER ONE

INTRODUCTION

Physical and Chemical Properties of 4-Vinyl-1-cyclohexene diepoxide

4-Vinyl-1-cyclohexene diepoxide (VCD) or 1,2-epoxy-4-(epoxyethyl) cyclohexane (IUPAC) is a colorless, water-soluble liquid. VCD is a small molecule with a molecular weight of 140.2 and its chemical formula is $C_8H_{12}O_2$. It has a specific gravity of 1.0986 at 20 °C, freezing point of -108.9 °C, boiling point of 227 °F, vapor pressure of 0.1 torr at 20 °C, open-cup flash point of 230 °F (110 °C), refractive index of 1.4787, and a viscosity of 7.77 centipoise at 20 °C. VCD has no chromophore and therefore, does not absorb in the visible or UV range. Additionally, VCD has four chiral carbons which can yield eight different diastereomers. Both of these characteristics make quantification of VCD difficult. Fortunately, VCD can be synthesized with a radioactive isotope of carbon which enables quantification of both VCD and its metabolites.

Production and Use of VCD

The major manufacturer of VCD in the United States is Union Carbide Corporation. However, the production volumes for VCD are not available (Union Carbide, 1978). It is manufactured by epoxidation of 4-vinylcyclohexene with peroxyacetic acid in an inert solvent (Wallace, 1964).

VCD is used as a chemical intermediate (e.g., for condensation with dicarboxylic acids) and as a monomer for preparation of polyglycols containing unreacted epoxy

groups or for homopolymerization to a three dimensional resin. It is also used as a reactive diluent for other diepoxides and for epoxy resins derived from bisphenol A and epichlorohydrin (IARC, 1976).

Human Exposure to VCD

The National Institute for Occupational Safety and Health (NIOSH) estimated that 70,000 workers were exposed annually to VCD. The National Occupational Exposure Survey (NOES), conducted by NIOSH between 1981 and 1983, estimated that 2,000 workers in the United States were potentially exposed to hazardous levels of VCD (NIOSH, 1988). Because of the potential for exposure, a threshold limit value/time-weighted average of 10 ppm (60 mg/m^3) for skin has been recommended by the American Conference of Governmental Industrial Hygienists (ACGIH, 1986).

Occupational exposure to VCD occurs primarily via inhalation during the manufacture of polyesters, coatings and plastics (Miller, 1978). Electron microscopists are also exposed to VCD present in the embedding media (Murray and Cummins, 1979). A study of the cancer epidemiology of this professional group may be in order, as many microscopists may have been exposed to VCD through inhalation and/or dermal absorption. However, these electron microscopists may have been simultaneously exposed to other toxic chemicals which would make the epidemiology study more difficult to evaluate. Also, information on other risk factors such as smoking, diet, alcohol consumption, and other life-style differences, would make the association of VCD exposure and increased cancer risk difficult even in the small population of electron microscopists.

Toxicology of VCD

When applied to the skin of humans, VCD causes mild-to-moderate skin irritation. When tested in rabbits, dermal application of VCD caused edema and redness comparable to a mild-to-moderate first-degree burn. Further, in guinea pigs, VCD resulted in sensitization of the skin (ACGIH, 1986). The percutaneous LD_{50} in rabbits was reported to be 0.68 g/kg (Weil et al., 1963). The LC_{50} of VCD in rats was 4580 mg/m^3 (800 ppm) (Hine and Rowe, 1963), while the oral LD_{50} was reported as 2.83 g/kg for rats (Weil et al., 1963).

Acute Toxicity

Acute toxicity of VCD has been reported by the National Toxicology Program (NTP, 1989). Short-term dermal toxicity studies were conducted by applying VCD in acetone for 14 days or 13 weeks to the skin or Oral gavage for 16 days or 13 weeks to Both genders of Fischer 344 rats and $B6C3F_1$ mice were used. These studies were designed to study the effect of different routes of exposure on target organ toxicity of VCD. Another objective of these studies was to select appropriate dose levels of VCD for subsequent chronic toxicity and carcinogenesis studies.

In the dermal exposure studies, rats of each sex received 0, 3.75, 7.5, 15, 30, or 60 mg/ml VCD in acetone, by dermal application, 5 days per week for 13 weeks. Mice of each sex were also administered 0, 0.625, 1.25, 2.5, 5, or 10 mg/ml on the same schedule as rats. There was no compound-related mortality seen in either species at any dose studied. The highest (two) dose levels resulted in compound related skin lesions at the application site for both species and genders. The lesions consisted of epithelial necrosis and ulceration, epidermal hyperplasia, hyperkeratosis, and sebaceous gland hyperplasia. In addition, ovarian atrophy, characterized by a decrease in the number of

primordial and secondary follicles, was observed in mice at the two highest doses of VCD (NTP, 1989; and Chhabra et al., 1990a). These results were consistent with the findings of an earlier study by Weil et al. (1963).

For the oral exposure studies, groups of rats and mice of each gender were administered 0, 62.5, 125, 250, 500 or 1000 mg/kg VCD in corn oil by gavage, 5 days per week, for 13 weeks (NTP, 1989; and Chhabra et al., 1990a). Compound-related mortality was observed only at the highest dose in both species. Loss in body weight was observed in both species at the two highest doses studied. VCD toxicity was primarily seen in the forestomach in rats and mice of both sexes. Diffuse hyperplasia and hyperkeratosis of forestomach stratified squamous epithelium was seen in all rats and mice except at 62.5 mg/kg dose. Degeneration of the testis, characterized by a decrease in the number of germinal epithelial cells was observed in male mice. In female mice, ovarian atrophy, characterized by a decrease in the number of follicles, was seen following exposure to three of the highest doses of VCD (NTP, 1989; and Chhabra et al., 1990a).

Chronic Toxicity

Numerous studies have demonstrated chronic toxicity of VCD in rodents. Dermal application of 16 mg VCD, 5 days a week for 1 year, resulted in squamous cell carcinomas in over half of the male mice exposed (Hendry et al., 1951). In another study, when 10% VCD in acetone was applied dermally to C3H mice for 21 months, 4/18 mice developed skin neoplasms (Weil et al. 1963). Van Duuren et al. (1963) reported development of neoplasms in 14/30 male mice exposed dermally to 10% VCD in benzene for 3 days a week for 1 year. Greater than 60% of these animals also had squamous cell carcinomas of the skin.

Recently, the National Toxicology Program conducted chronic toxicity testing for VCD (NTP, 1989; and Chhabra et al., 1990b). These studies focused on chronic dermal exposure because human exposure occurs primarily by this route. VCD (in acetone) was applied dermally to groups of 60 male and female Fischer 344 rats at doses of 0, 15, or 30 mg/rat, 5 days per week for 105 weeks. Similarily, groups of 60 $B6C3F_1$ mice of both sex were also exposed dermally to 0, 2.5, 5, or 10 mg/mouse of VCD on a schedule similar to rats for 105 weeks. The doses selected did not result in severe ulceration of the skin or life-threatening weight losses during the 13-week study. During the 2 year study, 10 animals from each group were killed at 15 months of dosing for toxicologic evaluation. Squamous cell carcinoma at or near the site of application was observed in two of the male rats in the high dose group. Also, hyperkeratosis and acanthosis of the skin was observed in rats receiving high dose of VCD. In contrast, one of the 10 female rats that received the 30 mg dose by dermal application, had squamous cell carcinoma of the forestomach.

In the VCD treated mice, non-neoplastic skin lesions included sebaceous gland hyperplasia, acanthosis, and hyperkeratosis. Squamous cell papillomas and carcinomas were observed in male and female mice at 5 and 10 mg VCD. Atrophy of the ovarian tissue was also seen in all VCD-treated female mice. Tubular hyperplasia of the ovarian surface epithelium was observed in 80% of female mice dosed with 5 mg and 100% of female mice given 10 mg VCD. At the high dose of 10 mg VCD, ovarian granulosa cell tumors were observed in 2 of 9 female mice, and ovarian papillary cystadenoma in 1 of 9 mice (NTP, 1989; and Chhabra et al., 1990b).

Results from the 2-year NTP study (1989) also indicate that body weights and survival rates were lower in mid and high dose groups of rats and mice. VCD was found to be carcinogenic to skin at the site of application in rats and mice of both genders. By

the end of the 2-year study, all treated rats and mice in the 5 and 10 mg dose groups had skin lesions which progressed to squamous cell carcinomas. Squamous cell carcinomas were the predominant skin tumor observed in both rats and mice. Basal cell adenomas and carcinomas were also observed in both species, but occurred more frequently in rats. These results are consistent with the findings from previous studies which have shown VCD to be a skin carcinogen in mice (Hendry et al, 1951; Van Duuren et al., 1963; and Weil et al. 1963).

Follicular atrophy and tubular hyperplasia of the ovary occurred in all VCD treated female mice. Benign and malignant neoplasms of the ovary occurred in the 5 and 10 mg dose groups of female mice. Also present at these two doses were benign and malignant granulosa cell tumors. A few of these granulosa neoplasms were malignant, hemorrhagic, and had metastasized to the lungs. Ovarian tumors were not seen in vehicle controls thereby indicating lack of spontaneous ovarian tumors (NTP, 1986). Also, ovarian neoplasms were not observed in female rats at the doses examined in this study. Therefore, it is apparent from the results that female mice are more susceptible to VCD induced ovarian tumors than are female rats.

Immunotoxity of VCD

The immunotoxic effects of VCD were studied by the National Toxicology Program in male $B6C3F_1$ mice following a 5 day dermal exposure at a dose range of 2.5 to 10 mg/mouse (NTP, 1989). No change in body weights and lymphoid organ weights (i.e., spleen, thymus, or mesenteric lymph nodes) were observed at the doses studied. However, VCD was immunosuppressive at 10 mg and to a lesser extent at the 5 mg dose. This was indicated by a decrease in peripheral lymphocytes, an *in vitro*

lymphoproliferative response study (phytohemagglutinin and concanavalin A), and suppression of antibody plaque-forming-cell response.

The mechanism of VCD induced immunotoxicity is currently unknown. However, Nishizuka et al. (1976) reported a relationship between immunosuppression and ovarian neoplasms in mice. They proposed that oocyte depletion stimulated an increase in gonadotropins, which subsequently promoted progression of ovarian neoplasms. It is interesting that ovarian atrophy, immunosuppression, and ovarian neoplasms occur in mice treated with VCD, but not rats. Further studies are required to determine if the immunosuppression from VCD is responsible or associated with the rare ovarian toxicity in mice.

Genetic Toxicity of VCD

A number of studies have demonstrated the mutagenic activity of VCD in *Salmonella typhimurium,* particularly with base-substitution indicator strains TA100 and TA1535 with or without S9 fractions (Murray and Cummins, 1979; Wade et al., 1979; Frantz and Sinsheimer, 1981; Turchi et al., 1981; Mortelmans et al. 1986, and NTP, 1989). When *Salmonella typhimurium* strains TA98, TA100, and TA1535 were exposed to 100-10,000 μg VCD per plate a dose-related increase in revertant colonies was observed both in presence and absence of metabolic activation (NTP, 1989).

In V79 Chinese hamster lung cells, Turchi et al. (1981) showed that VCD induced point mutations and chromosomal abnormalities (anaphase bridges and micronuclei). Further, VCD caused gene reversions, conversions, and mitotic crossing-over in *Saccharomyces cerevisiae* (Bronzetti et al., 1980). In both of these studies, VCD caused mutations in the absence of metabolic activation with S9 fraction. VCD also caused cytogenetic damage in cultured Chinese hamster ovary cells, producing sister

chromatid exchanges and chromosomal aberrations. Collectively, these studies indicate that VCD is a direct acting mutagen inducing reverse gene mutations in both frame-shift and base-substitution strains of *Salmonella typhimurium* in the absence of S9 metabolic activation.

Although VCD has been shown to be mutagenic both in the presence and absence of S9 fraction, one of its metabolites 4-ethylenoxycyclohexane-1,2-diol did not induce gene mutations in *Salmonella typhimurium* strain TA100 or in V79 Chinese hamster lung cells (Gervasi et al., 1981). However, Turchi et al. (1981) reported that 4-ethylenoxycyclohexane induced anaphase bridge formation and micronuclei in V79 Chinese hamster lung cells at a concentration of 2.0 mM.

Although the mutagenic mechanism of VCD has not been identified, its mutagenic activity in the absence of metabolic activation suggests covalent binding of epoxides to DNA (Oesch, 1982). VCD has two epoxide groups capable of alkylating nucleophilic sites on cellular macromolecules, such as nucleoproteins and DNA, similar to propylene oxide (Djuric et al., 1986) and other alkyl epoxides (Citti et al., 1984). VCD may also be a bifunctional alkylating agent, capable of forming interstrand or intrastrand cross-links of DNA or DNA-protein cross-links. Agents of this type are known to be mutagenic (Anderson et al., 1978).

Metabolism and Disposition of VCD

The *in vitro* metabolism of VCD was investigated in rabbit liver microsomal incubations (Watabe and Sawahata, 1976). The metabolites of VCD identified were 1,2-dihydroxy-4-vinylcyclohexane oxide, 4-(1',2'-dihydroxyethyl)-1-cyclohexane oxide, and 4-(1',2'-dihydroxyethyl)-1,2-cyclohexane diol (Figure 1.1). Control experiments using heat-denatured microsomes indicated metabolite formation was enzymatic. Further, the

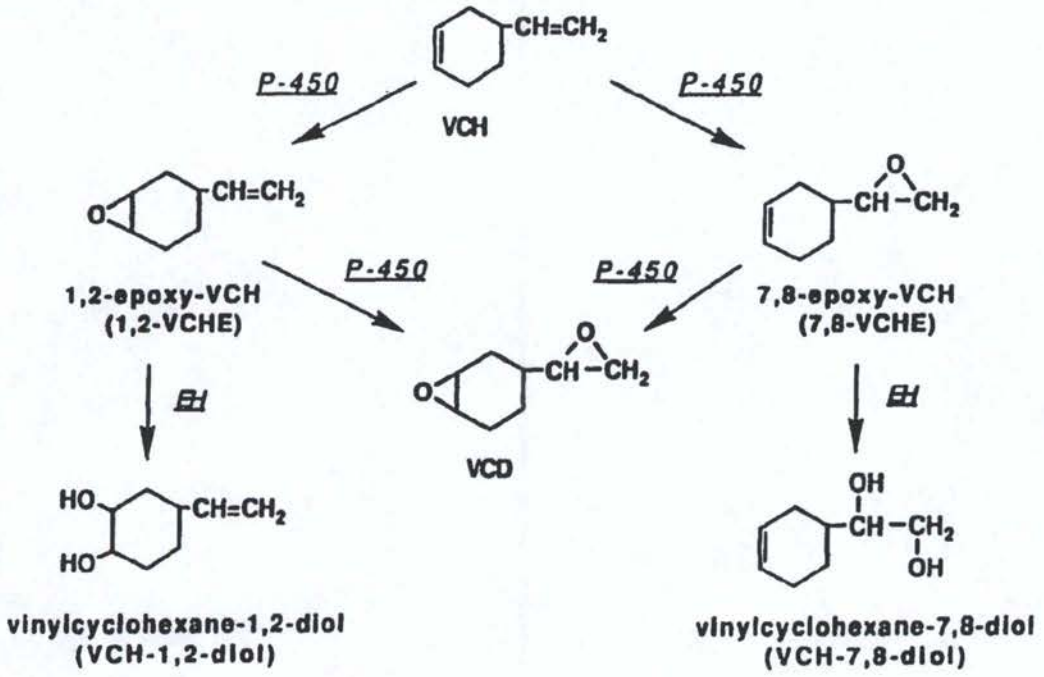

Figure 1.1 Proposed metabolic pathway of 4-vinyl-1-cyclohexene diepoxide.

authors suggested microsomal epoxide hydrolase was responsible for the formation of these metabolites (Watabe and Sawahata, 1976).

Conjugation with glutathione (GSH) is another pathway for metabolism of VCD (Figure 1.1). Epoxides and arene oxides are good substrates for GSH conjugation. Hayakawa et al. (1975) examined VCD among 50 different epoxides and arene oxides to form GSH conjugates in sheep liver cytosol. GSH conjugation of VCD was not detected under these experimental conditions. However, styrene oxide, cyclohexene oxide (Hayakawa et al. 1975), and 3,4-epoxyvinylcyclohexane (Boyland and Williams, 1965), which are structurally similar to VCD, were good substrates for GSH.

Although, Hayakawa et al. (1975) reported VCD was not a substrate for GSH conjugation in sheep liver cytosol, Giannarini et al. (1981) examined the level of GSH following intraperitoneal administration of 500 mg/kg VCD, 4-vinylcyclohexene monoxide (VCM), or 4-vinylcyclohexene (VCH) in male Swiss albino mice. VCD and VCM depleted 96% of liver GSH within 2 hr while VCH had a similar effect in 4 hr. These results suggest that depletion of liver GSH is most likely a result of formation of GSH conjugates with parent compound and/or their metabolites (Giannarini et al., 1981).

Previous studies have shown that female $B6C3F_1$ mice are more susceptible to VCD-induced ovarian toxicity than female Fischer 344 rats (NTP, 1989; Chhabra et al., 1990a; Chhabra et al., 1990b; and Smith et al., 1990b). To investigate the species difference Sipes et al., (1989) examined the disposition of a single dermal application of ^{14}C-VCD in female Fischer 344 rats and $B6C3F_1$ mice. Rats were dosed with 100 ul of ^{14}C-VCD in acetone (50 mg; 20 μCi) and mice with 10 ul of the same solution (5 mg; 2.0 μCi). Only 30% of the applied dose was absorbed over a 24 hr period in both species. The remainder of the dose was present in the charcoal trap above the application site in both species. By 24 hr, 70-80% of the absorbed dose (30%) had been excreted in

both species. The major route of elimination in both species was via the urine, which accounted for approximately 75% of the absorbed dose. Radiolabel present in the feces and expired air accounted for less than 5% of the absorbed dose in both species. Therefore, no major differences in the amount or route of elimination of radiolabel was observed between the species.

At 24 hr following dermal application of ^{14}C-VCD, only 5% of the applied radioactivity remained in the tissues in both species. Liver, muscle, and adipose tissue contained 0.5-1.6% and 1.2-2.9% of the dose absorbed in rat and mouse tissues, respectively. Tissue-to-blood ratios ranged from 0.3 to 1.5 in rats and from 0.8 to 2.8 in mice. The tissue-to-blood ratio in mouse ovarian tissue was 5-fold greater compared to ovarian rat tissue (Sipes et al., 1989). This indicates selective retention of VCD and or its metabolites by mouse ovarian tissue and may, in part, explain the species difference in susceptibility to VCD induced ovarian toxicity.

Many studies have shown that the ovary is one of the target tissues for VCD-induced toxicity (NTP, 1989; Chhabra et al., 1990a; Chhabra et al., 1990b; and Smith et al., 1990b). However, the role of the ovary in the metabolism of VCD has not been studied. Microsomal epoxide hydrolase as well as cytosolic glutathione-S-transferases, both proposed to be involved in the metabolism of VCD, are present in the ovary (Mukhtar et al., 1978). For example, Mukhtar et al. (1978) utilized intraovarian injection and demonstrated that the ovary has the capacity to metabolize benzo(a)pyrene oxide to benzo(a)pyrene 4,5-dihydrodiol. Another study reported the production of diol, quinone, and phenolic metabolites of 7,12-dimethylbenz(a)anthracene in rat ovarian microsomal incubations (Bengtsson et. al., 1983). Future studies are necessary to evaluate the ovaries capacity to metabolize or detoxify VCD. These studies will lead to a better understanding as to why one species is more susceptible to VCD-induced ovotoxicity.

Reproductive Toxicity of VCD

The biological mechanisms of reproduction are complex. This process begins with gametogenesis, continues through gamete interaction, implantation, embryonic development, growth, parturition, and is complete with a sexually mature animal. Reproductive toxicants can produce an adverse effect on one or several of these complex mechanisms. Summarized below is an overview of the female reproductive cycle, mechanisms through which reproductive toxicants can act, and a few examples of xenobiotics which interrupt this complex but essential process.

The ovary is the primary reproductive organ in female mammals. It is responsible for oogenesis and the synthesis of steroids. In mammals, oogenesis is completed before birth. Each oocyte is incorporated into a follicle, which consists of the oocyte surrounded by a single layer of granulosa cells. Females are born with a finite number of immature ovarian follicles called primordial follicles (Hirshfield, 1991). The development of a primordial follicle to an ovulatory follicle involves several stages of maturation through a primary, secondary, and tertiary follicle, followed by development into a pre-ovulatory follicle (Figure 1.2).

Reproductive toxicants that destroy follicles at different stages of development will have different impacts on reproduction. For example, compounds which destroy large growing follicles, or antral follicles, will cause an immediate decrease in fertility. However, after exposure to the toxicant, oocytes from the growing pool will repopulate the preovulatory pool and fertility will resume. If follicles in the growing pool are destroyed, fertility will decline, perhaps even after removal of the ovotoxicant. The extent to which fertility declines will depend on the number of growing oocytes destroyed and the time it takes to repopulate from the pool of primordial and primary follicles. However, once the pool of primordial follicles is destroyed and the pool of

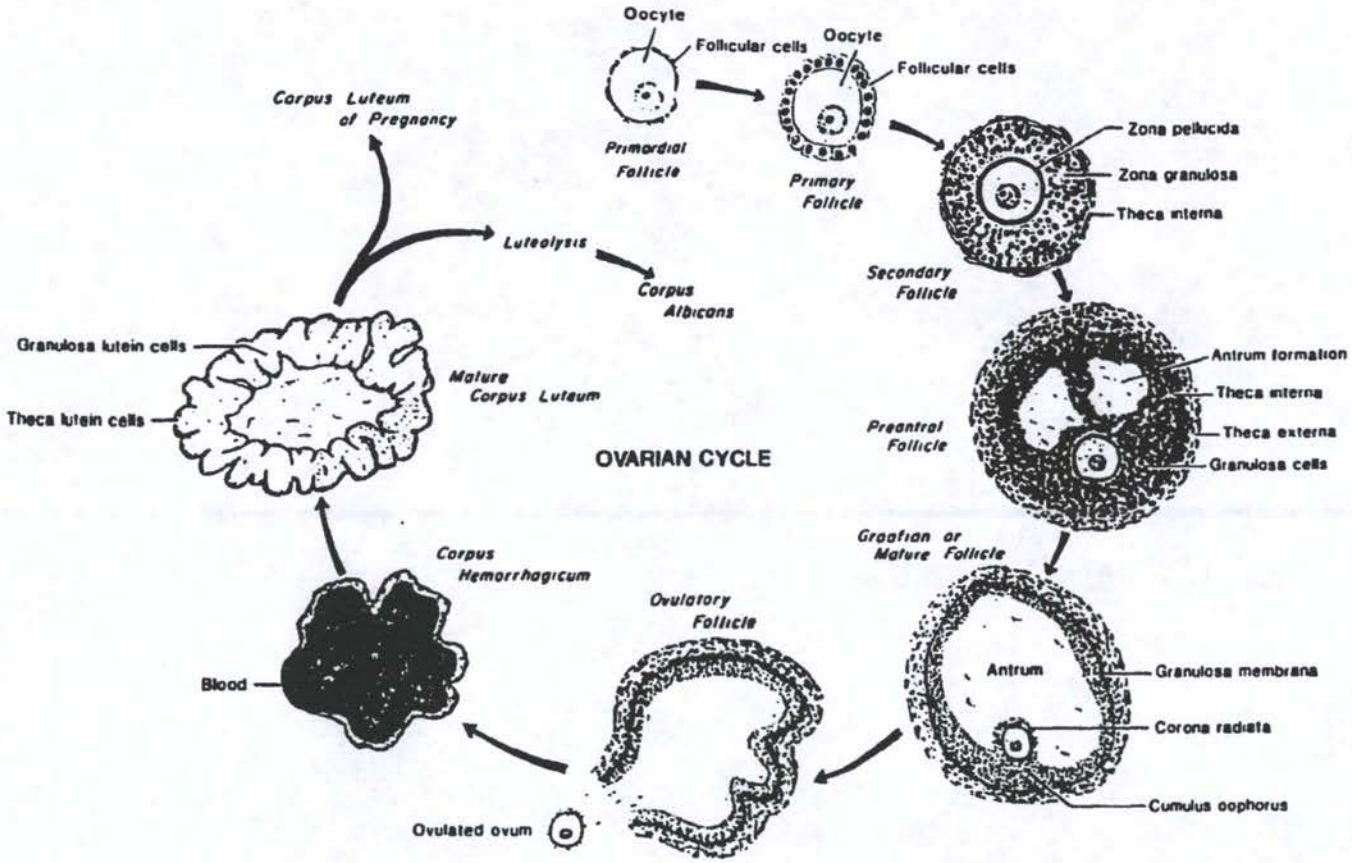

Figure 1.2 The mammalian ovarian cycle. Figure taken directly from Hadley (1984).

growing and antral follicles can no longer be supported, fertility ceases completely and permanently. Primordial follicles or small oocytes have been shown to be the most vulnerable cells in the ovary to chemicals and ionizing radiation (Jull, 1973).

In addition to the primordial follicles, the reproductive cycle is vulnerable at many points to the influence of xenobiotics. Ovotoxicants can be either direct-acting or indirect-acting on one or more of the reproductive processes. The direct-acting toxicants are structurally similar to endogenous molecules such as; hormones, nutrients, etc. For example, oral contraceptives act primarily by suppression of gonadotropin secretion. Other direct-acting toxicants act by virtue of their nonspecific chemical reactivity (ie, alkylating agents, chelators, and denaturants).

Indirect acting reproductive toxicants require metabolic activation within the organism to exert their toxic effect or act by modifying physiological mechanisms. Examples of indirect-acting reproductive toxicants that require metabolic activation include cyclophosphoramide (Plowchalk and Mattison, 1991), polycyclic aromatic hydrocarbons (Mattison, 1979), and diethylstilbesterol (Metlzer, 1976). In contrast, compounds such as halogenated polycyclic hydrocarbon pesticides act by modifying physiological control mechanisms, such as enzyme activity. A change in enzyme activity may alter the rate of steroid synthesis or clearance, which in-turn, can alter hormonal feedback loops responsible for control of reproductive processes.

Many xenobiotics are toxic to the ovary because of their ability to destroy oocytes (Dobson and Felton, 1983). Studies with chemicals which cause ovarian cancer have shown that destruction of oocytes precedes the formation of tumors. Mattison (1979) demonstrated that dimethylbenzanthracene (DMBA), benz(a)pyrene (BP), and 3-methylcholanthrene (MC) destroyed oocytes to a greater extent in C57B1/6N and DBA/2N mice compared to Sprague Dawley rats. In another study Mattison and

Thorgeirsson (1979) investigated the genetic differences in C57B1/6N and DBA/2N mouse ovarian aryl hydrocarbon hydroxylase activity and changes in oocyte numbers following treatment with 3-MC. The activity of aryl hydrocarbon hydroxylase in C57B1/6N was induced over 3-fold by 3-MC treatment whereas no change was observed in DBA/2N mice. Cleary, these studies suggest that one reason for this species and/or strain difference may be due to variations in enzyme levels responsible for the bioactivation of the polycyclic aromatic hydrocarbons in the ovary.

Many studies have clearly demonstrated that polycyclic aromatic hydrocarbons need to be bioactivated to reactive epoxides before they are ovotoxic (Mattison and Thorgeirsson, 1979; Shiromizu and Mattison, 1984; and Shiromizu and Mattison, 1985) For example, Shiromizu and Mattison (1985) showed that intraovarian injection of DMBA or 3-MC destroyed oocytes in C57B1/6N and DBA/2N mice. Further, when mice were pretreated with α-napthoflavone, a competitive inhibitor of the enzymes necessary for bioactivation of DMBA and 3-MC, oocyte destruction was inhibited (Shiromizu and Mattison, 1985). These results cleary demonstrate the ovary has the enzymes necessary to bioactivate DMBA and 3-MC to their reactive metabolites.

Another compound which is ovotoxic and structurally similar to VCD is 4-vinyl-1-cyclohexene (VCH) (Figure 1.3). The toxicity of VCH was studied in B6C3F_1 mice and Fischer 344 rats by the NTP (NTP, 1986; and Collins and Mannus, 1987). The toxicity of VCH administered by oral gavage for 13 weeks was examined in both sexes of B6C3F_1 mice and Fischer 344 rats (Collins and Manus, 1987). The dose of VCH ranged from 0-800 mg/kg for rats and 0-1200 mg/kg in mice. VCH induced a dose-dependent hyaline droplet degeneration of the proximal tubule in male rats only. Another histologic effect observed from VCH treatment was a reduction in numbers of primary and mature follicles in the ovaries of all high dose female mice (Collins, 1987).

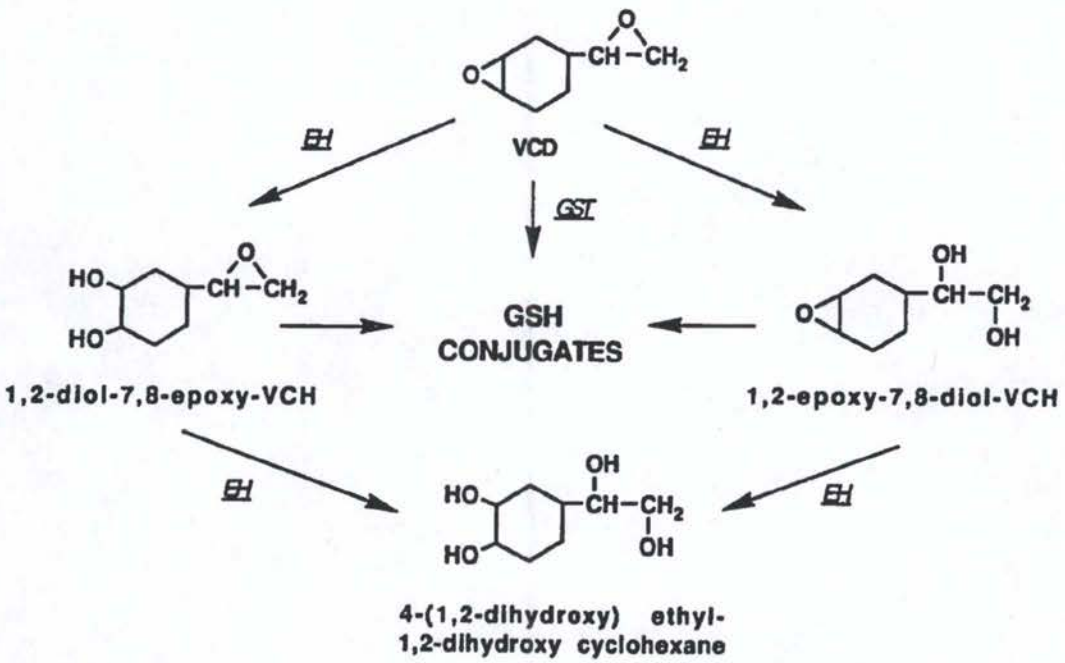

Figure 1.3 Proposed metabolic pathway of 4-vinyl-1-cyclohexene.

Based on the results of the 13 week study, the chronic toxicity of VCH was studied in both genders of $B6C3F_1$ mice and Fischer 344 rats (Collins et al., 1987). Both species were dosed orally with VCH at 0, 200 or 400 mg/kg, 5 days per week for 103 weeks. Female mice treated with VCH had a significant increase in ovarian neoplasms including mixed benign tumors and granulosa cell tumors and/or carcinomas. There was no increase in the incidence of ovarian tumors observed in rats at any dose of VCH examined. However, because of the extensive and early mortality at both doses tested, this study was considered to be an "inadequate study of carcinogenicity" by the NTP (1986).

To further investigate the species difference observed in VCH-induced ovotoxicity, Smith et al. (1990a) examined the disposition of a single intraperitoneal dose of VCH (800 mg/kg) in female $B6C3F_1$ mice and Fischer 344 rats. The animals were were killed at various times over a 6 hr period following administration of VCH, and plasma analyzed for parent compound and its metabolites. VCH-1,2-epoxide (VCM), an oxidative metabolite of VCH, was detected in the plasma from mice but not rats (Smith et al., 1990a). These results provided the rationale for studying the metabolism of VCH in hepatic microsomes from both species. The rate of VCH epoxidation was determined to be 6.5-fold greater in mouse microsomes compared to the rat (Smith et al., 1990a). These metabolism rates are consistent with reported values in the rat (Watabe et al., 1981) and in the mouse (Gervasi et al., 1981).

Previous studies have shown that VCH caused oocyte destruction and ovarian neoplasms in female $B6C3F_1$ mice but was not ovotoxic in Fischer 344 rats (NTP,1986; Collins and Mannus, 1987; and Collins et al., 1987). To further investigate this species difference, Smith et al. (1990b) examined the ovotoxic potency of VCH and its reactive epoxide metabolites in female $B6C3F_1$ mice and Fischer 344 rats. Doses of 100, 400,

and 800 mg/kg VCH were selected based on the subchronic NTP study (NTP, 1986, and Collins and Manus, 1987). Because epoxides are more reactive, doses of 10, 40, and 80 mg/kg of the diepoxide (VCD) were used, and intermediate doses of 42.5, 170, and 340 mg/kg were selected for the monoepoxides. The chemicals were administered by intraperitoneal injection daily, for 30 days (Smith et al., 1990b).

The results of the study indicate that VCH caused a dose and time dependent loss of small oocytes in the ovaries of mice, but not in rats. However, treatment with VCH 1,2-epoxide and VCD produced a dose dependent decrease in small oocytes in both species. The diepoxide was the most potent compound tested at depleting small oocytes in both species (Figure 1.4). The ED_{50} for VCH-1,2-epoxide and VCD were at least 2-3 fold lower in the mouse compared to the rat. These results clearly indicate that the potency of the monoepoxides of VCH and VCD to deplete small oocytes is much greater than VCH. Further, the mouse was more sensitive to the ovotoxic effects of all the compounds tested when compared to the rat (Smith et al., 1990b).

Consistent with the above results, VCH-1,2-epoxide was identified in the plasma of mice, but not rats following a single intraperitoneal dose of VCH (Smith et al. 1990a). Also, the rate of VCH epoxidation was much greater in mouse microsomes compared to the rat (Smith et al. 1990a). Although, the monoepoxide destroyed small oocytes in both species (Smith et al. 1990b), VCH was only ovotoxic in the mouse (NTP, 1986; Collins and Manus, 1987; Collins et al., 1987; and Smith et al., 1990a and 1990b). These results indicate that the female mouse has a significantly greater capacity to bioactivate VCH to its ovotoxic metabolite than does the rat (Smith et al., 1990a and 1990b).

Relationship Between Dose and Small Oocyte Counts in Ovaries from Mice and Rats Treated with VCH, 1,2-VCHE, 7,8-VCHE, and VCD

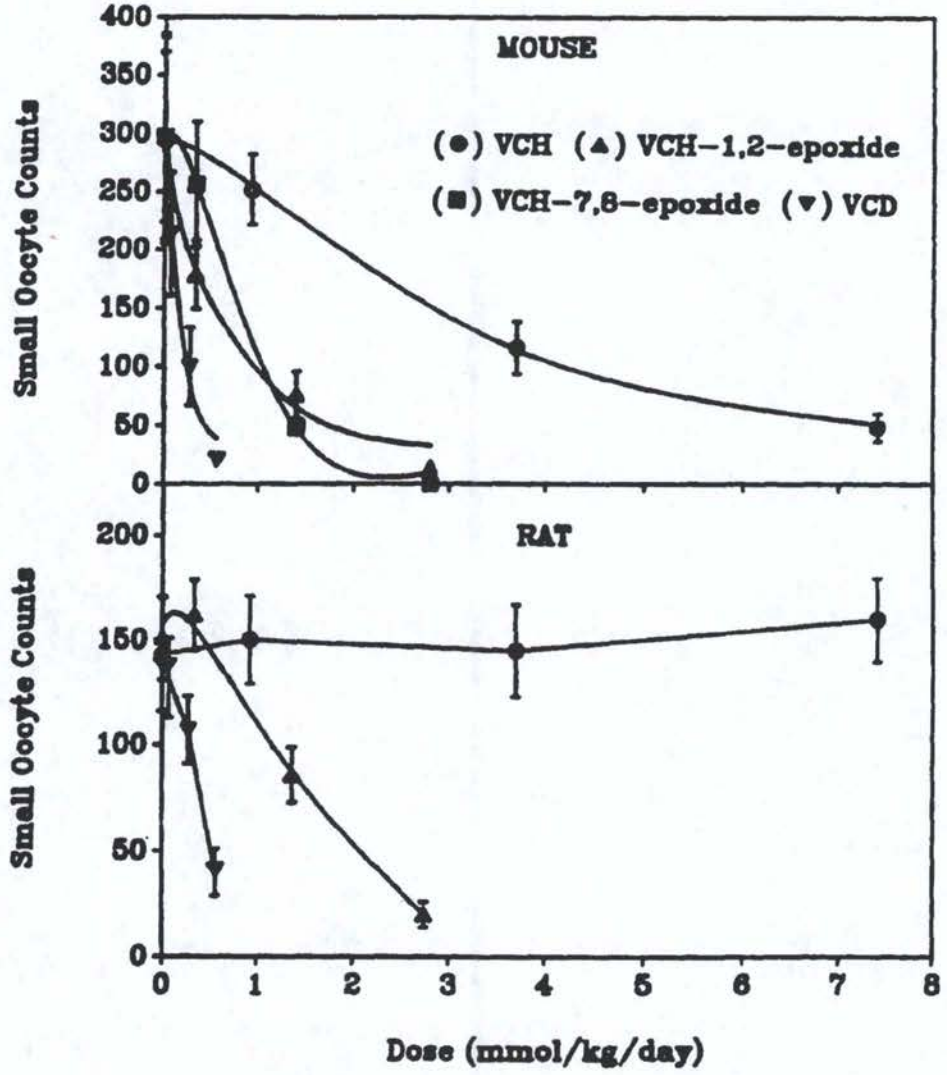

Figure 1.4 Comparison of the dose-response relationship in the reduction in small oocyte counts in the ovaries of rats and mice treated with VCH or VCH epoxides i.p. for 30 days. Each point represents the mean of 4-10 animals ± S.D. Figure taken directly from Smith et al., (1990b).

STATEMENT OF PROBLEM

VCD is a known carcinogen, a direct acting mutagen, and a ovarian toxicant. Since thousands of industrial workers are exposed to VCD in the United States, it is important to better understand the disposition and toxicity of VCD. Previous studies have shown female B6C3F$_1$ mice are more susceptible to ovarian toxicity caused from VCD than are Fischer 344 rats. The basis for the species difference in susceptibility to VCD-induced ovarian toxicity in rats and mice is unknown. Understanding the mechanism for this species difference is necessary to determine which species would best predict the toxicity of VCD in humans. It is proposed that one species may be more efficient at detoxification of VCD. A greater degree of detoxification of VCD by one species, would explain its decreased susceptibility to its ovotoxic effects. The studies presented in this dissertation were designed to test the hypothesis that: female mice are more susceptible to VCD-induced ovarian toxicity compared to female rats because of differences in the metabolism and/or disposition of VCD between the species. The results of this research will lead to a better understanding of the mechanisms involved in VCD toxicity.

OBJECTIVES

The objective of this research was to elucidate the basis for the species difference in susceptibility to VCD-induced ovarian toxicity in female Fischer 344 rats and B6C3F$_1$ mice. To address this objective this dissertation is divided into chapters as outlined below.

The second chapter, "The Disposition and Metabolism of 4-Vinyl-1-Cyclohexene Diepoxide in Female B6C3F$_1$ Mice and Fischer 344 Rats", compares the disposition of a single intraperitoneal dose of ^{14}C-VCD in the rat and mouse. Specifically, the route and rate of elimination of VCD and its metabolites were compared in both species. Also to identify specific tissues which may be more prone to VCD toxicity, the tissue distribution of VCD and/or its metabolites was investigated. To investigate the metabolism of VCD, urine was collected from each species following a single dose of ^{14}C-VCD and analyzed by HPLC.

The third chapter, "Toxicokinetics and Biliary Excretion of 4-Vinyl-1-Cyclohexene Diepoxide in Female Fischer 344 Rats and B6C3F$_1$ Mice", further characterizes the disposition of VCD by determining the toxicokinetic parameters of a single intravenous dose of ^{14}C-VCD in both species. Also, to further our understanding of the elimination process, the biliary excretion of a single intravenous dose of ^{14}C-VCD and its metabolites was determined. Both of these studies are essential to understanding the metabolism and elimination process of xenobiotics.

The fourth chapter, "*In Vitro* Metabolism of 4-Vinyl-1-Cyclohexene Diepoxide and its Modulation of Glutathione Levels in Female Fischer 344 Rats and B6C3F$_1$ Mice", characterizes the activity of microsomal epoxide hydrolase toward VCD in both species. Also the effect of a single dose of VCD on glutathione levels in the liver and ovary was determined. Many epoxides are detoxified by their conjugation with glutathione. Therefore, depletion of glutathione may render certain tissues more susceptible to VCD toxicity. Differences in the activity of detoxification enzymes toward VCD, or in availability of cosubstrates necessary for the detoxification process, may contribute to the difference in the ovotoxicity observed between rats and mice.

CHAPTER 2

DISPOSITION AND METABOLISM OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE IN FEMALE FISCHER 344 RATS AND B6C3F$_1$ MICE

INTRODUCTION

4-Vinyl-1-cyclohexene diepoxide (VCD) is a colorless, water-soluble liquid. It is manufactured by epoxidation of 4-vinylcyclohexene with peroxyacetic acid (Wallace, 1964). VCD has been used as a chemical intermediate in condensation reactions with dicarboxylic acids and as a monomer for preparation of polyglycols containing nonreactive epoxy groups (IARC 1976). Numerous studies have shown VCD to be a direct acting mutagen. VCD caused reverse gene mutations in both frame-shift and base-substitution strains of *Salmonella typhimurium* in the absence of S9 metabolic activation (Murray and Cummins, 1979; Frantz and Sinsheimer, 1981; Mortelmans et al. 1986; and NTP, 1989). Human exposure to VCD occurs primarily by inhalation or dermal contact in occupations involving the manufacture of polyesters, coatings and plastics (Miller, 1978). In the mid-1980's, the National Occupational Exposure Survey estimated that 70,000 workers in the United States are potentially exposed to VCD (NIOSH, 1988). Because of the potential for human exposure and lack of carcinogenicity data, NIOSH nominated VCD for study by the National Toxicology Program.

Despite the genotoxicity data and the potential for human exposure, there are limited studies describing the metabolism and disposition of VCD. Watabe and Sawahata (1976) investigated the *in vitro* metabolism of VCD in rabbit liver microsomes. Hydrolysis of one or both of the epoxides of VCD was observed, resulting in the formation of corresponding diols (Watabe and Sawahata, 1976). The hydrolytic reaction was enzymatic, and suggests a possible role of epoxide hydrolase for the formation of the diols.

The NTP conducted dermal application studies on male and female F-344 rats and B6C3F_1 mice over a 2 yr period (NTP, 1989). VCD showed carcinogenic activity for male and females of both species as shown by squamous and basal cell carcinomas of

the skin. Further, VCD was ovotoxic to mice as indicated by ovarian atrophy, decreased number of follicles, and ovarian neoplasms. VCD was not found to be ovotoxic to rats by any route or dose tested in this study (NTP, 1989; and Chhabra et al. 1990a and 1990b). However, Smith et. al. (1990b) reported that one intraperitoneal administration of VCD caused ovarian toxicity in both rats and mice.

The species difference in ovotoxicity may be related to the metabolism of VCD and/or its route of elimination. To investigate this possibility, the disposition of VCD following a single dermal application of ^{14}C-VCD was determined in female B6C3F$_1$ mice and Fischer 344 rats (Sipes et al., 1989). Only 30% of the applied dose was absorbed over a 24 hr period in both species. At 24 hr, 70-80% of the absorbed dose was excreted from the body. The major route of elimination in both species was via the urine (75% of the absorbed dose). The results indicate no major differences in the route of elimination between the two species. However, the tissue-to-blood ratio in mouse ovarian tissue was 5-fold greater compared to the rat ovarian tissue. The authors suggest this difference in ovarian tissue-to-blood ratio may in part explain the species difference in susceptibility to VCD-induced ovarian toxicity (Sipes et al., 1989). Rats were less susceptible tothe ovarian toxicity than mice.

Since the reason for the species difference in susceptibility to VCD-induced ovotoxicity has not been fully addressed, the following studies were undertaken to investigate differences in the disposition and/or metabolism of VCD between the female rat and mouse. A species difference in the disposition and/or metabolism of VCD could explain, in part, the species difference in susceptibility to VCD ovarian toxicity.

MATERIALS AND METHODS

Animals

Adult female B6C3F$_1$ mice (19-22g) and female Fischer 344 rats (150-170g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Rats were housed in hanging wire cages and mice in polycarbonate plastic pans with hardwood chip bedding. The animals were allowed free access to food (Teklad 4% mouse/rat; Harlan Teklad, Madison, WI) and water. The animals were maintained on a 12 hr light/dark cycle at an ambient room temperature of 21-22°C. The animals were acclimated for at least 7 days prior to use in the toxicokinetic studies. For the biliary excretion studies, jugular vein cannulated female Fischer 344 rats (150-170g) were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA). The rats were individually housed in metabolism cages in the same environment as above for at least 2 days prior to use in the biliary excretion studies. The jugular cannula was rinsed daily with saline and 100 ul of heparin-saline (1:1) solution was used to fill the lumen of the cannula to prevent coagulation prior to use.

Chemicals

4-vinylcyclohexene diepoxide and urethane were purchased from Aldrich Chemical Co. (Milwaukee, WI). The chemical purity of unlabeled VCD was >98% as determined by GC-MS. Radiolabeled ^{14}C-4-vinylcyclohexene diepoxide (sp. act. 6.0 mCi/mmol) was obtained from Chemsyn Science Laboratories (Lexena, KS). ^{14}C-VCD was purified by straight-phase HPLC (semi-preparatory column) and the radiochemical purity was determined to be >98% by straight-phase HPLC (analytical column). The chemical purity of the purified ^{14}C-VCD was also quantified by GC-MS and determined

to be >98%. CarboSorb^{R} and FLO SCINT™ II cocktail were obtained from Packard Instruments (Chicago, IL). Universol™ Cocktail was purchased from ICN Radiochemicals (Irvine, CA). Phenolphthalein glucuronide, p-nitrophenyl sulfate, lyophilized ß-glucuronidase type VII-A (from *Escherichia coli*) and sulfatase type V (from limpets) were purchased from sigma Chemical Co. (St. Louis, MO). All other chemicals and chromatography solvents used were either analytical grade or HPLC grade.

Chromatography

^{14}C-VCD was purified using a 10 µm Phenomenex Partisil^{R} silica semi-preparative column (250 x 10 mm). The mobil-phase was an isocratic solution of hexane:isopropanol (98:2), at 4 ml/min for 30 min. Radioactivity was analyzed by a flow scintillation detector (Radiomatic Instruments, Meriden, CT), at a combined flow rate of 8 ml/min (1:1 ratio of FLO SCINT™ II cocktail:mobile phase). Appropriate fractions containing ^{14}C-VCD were directly collected from the HPLC, between the column and the scintillation detector. The ^{14}C-VCD fractions were pooled and solvents removed by rotory evaporation. Purified ^{14}C-VCD was reconstituted in 100% ethanol and stored at -70 °C and used within 14 days.

The chemical purity of unlabeled and radiolabeled VCD was determined using a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA). The column used was a 0.32 mm x 10 M RSL-300 capillary column (Alltechs Associates; Deerfield, IL) with a flame ionization detector (FID). The FID gas flow rates for H_2, N_2, and air were 30 ml/min, 30 ml/min, and 240 ml/min, respectively (Smith et al., 1990a). The injector and detector tempertures were maintained at 200°C, and 300°C, respectively. The oven

temperature was constant at 40°C for 2 min, then ramped at 10°C/min for 11 min. Under these conditions VCD eluted at 7.6 min ±0.2 min.

Separation of ^{14}C-VCD and its metabolites from urine samples was accomplished using a 7 μm Phenomenex CN (Zorbax^{R}) analytic HPLC column (4.6 x 250 mm). The mobile phase consisted of 90:10 hexane:isopropanol at time zero, a gradient to 50:50 hexane:isopropanol at 20 min, and a further gradient to 100% methanol at 60 min. For quantification, radioactivity was analyzed using a model B flow scintillation detector (Radiomatic Instruments, Meriden, CT), at a combined flow rate of 3 ml/min (2:1 ratio of FLO SCINT™ II cocktail:mobile phase).

Disposition Study

Animals were acclimated to metabolism cages 24 hr prior to dosing. ^{14}C-VCD (100 mg/kg; i.p.) was administered to female Fischer 344 rats (10 μCi/rat) and female B6C3F$_1$ mice (5.5 μCi/mouse). Following administration of dose, urine and feces were collected at 6, 12, 24, 36, 48, 60, and 72hr. At the end of 72 hr, animals were killed by carbon dioxide inhalation and total necropsy was performed. Aliquots of tissue samples (100-200 mg) were oxidized using a Packard Model 306 Sample Oxidizer (United Technologies; Downers Grove, IL), and analyzed for total radioactivity using a Beckman (LS 5000 TDC) scintillation counter. Feces were combusted to $^{14}CO_2$ and counted for radioactivity as described for tissues. Aliquots of urine (0.1 ml) were analyzed for total radioactivity using a scintillation counter. In addition, urine samples were also analyzed for parent compound and its metabolites using HPLC.

To determine the amount of ^{14}C-VCD and/or its metabolites which was excreted in expired air, female Fischer 344 rats were acclimated to glass metabolism cages 24 hr prior to dosing. For collection of expired air, a vacuum pump pulled air through the

cage, then through three ice-cooled traps. The first trap contained 2-methoxyethyl ether for collection of volatile organic compounds, the second and third traps each contained Carbo-Sorb^{R} ethylene glycol (2:1 v/v) for collection of $^{14}CO_2$. At various times, traps were changed and aliquots from each were analyzed for radioactivity by liquid scintillation counting.

Enzyme Assays

Urine from rats and mice dosed with ^{14}C-VCD was incubated with 800 units ß-glucuronidase (from *Escherichia coli*) or 25 units sulfatase (from limpets). Assay procedure and conditions were taken from Sigma insert which accompanies enzymes. To determine enzyme activity, standard curves using phenolphthalein glucuronide and p-nitrophenyl sulfate were used.

Mass Spectra Analysis

Mass spectrometry data was acquired on a Finnigan (4500) mass spectrometer equipped with a thermal pneumatic nebulizer coupled with a momentum separator (Upjohn Corp., Kalamazoo, MI). The instrument was operated in full-scan positive ion chemical ionization mode, using ammonia as the reagent gas.

RESULTS

Excretion of Radiolabel

The cumulative excretion of radiolabel in expired air following a single intraperitoneal injection of ^{14}C-VCD in female Fischer 344 rats is shown in Figure 2.1.

Excretion of Radiolabel in Expired Air from Female Fischer 344 Rats Following ^{14}C-VCD Administration

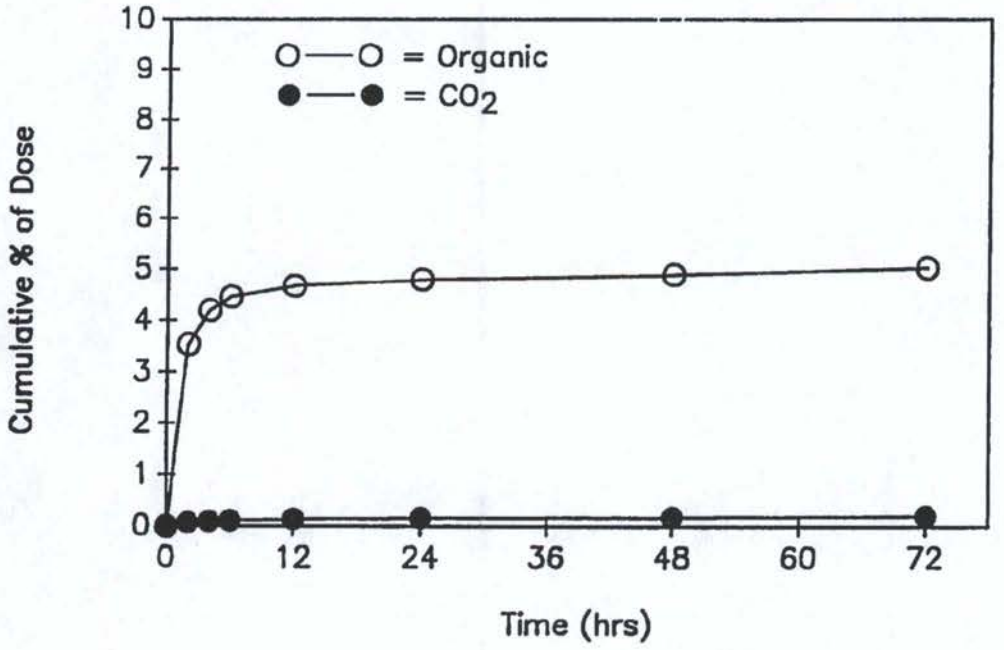

Figure 2.1 Cumulative excretion of radiolabel in expired air following a single intraperitoneal injection of ^{14}C-VCD (100 mg/kg; 10 µCi). Data represents the mean of two animals.

By 72 hr, approximately 5% of the dose was excreted in the expired air. The majority of the expired radiolabel was collected in the organic trap, which suggests it was most likely parent compound due to its higher volatility compared to its metabolites. A negligible amount of radiolabel was collected in the CO_2 trap, indicating ^{14}C-VCD was not metabolized to $^{14}CO_2$.

Urinary excretion was the primary route of elimination of ^{14}C-VCD derived radiolabel in both species (Figures 2.2 and 2.3). Within 72 hr, approximately 80% and 90% of the administered radiolabel was excreted in the urine of mice and rats, respectively. A greater amount of the radiolabel was excreted in the rat within 24 hr as compared to mice, and this difference was even more pronounced at earlier time points. For example, at 6 hr the rats had excreted approximately 70% of the radiolabel in the urine, compared to 30% in the mice. Elimination of ^{14}C-VCD derived radioactivity in the feces was a minor route of excretion and accounted for 4-5% of the dose in both species.

Tissue Distribution of Radiolabel

The tissue distribution of radiolabel was examined in female Fischer 344 rats and B6C3F$_1$ mice at 72 hr following a single intraperitoneal administration of ^{14}C-VCD. At 72 hr, all tissues examined in both species contained less than 1% of the dose. Total radioactivity present in all tissues examined (% of dose) was 3.9% ± 0.52% in the rat , and 6.8% ± 0.40% in the mouse. All tissues in rats had a tissue:blood ratio (for total ^{14}C) of less than one (Figure 2.4). In the mouse, all tissues examined has a tissue:blood ratio equal to, or more than one (Figure 2.5). The highest tissue concentration of radiolabel was detected in the liver, spleen, and kidney in both species. In the mouse, the liver had a 3-fold higher concentration of radiolabel than the blood. The tissue:blood

Excretion of Radiolabel in Expired Air from Female Fischer 344 Rats Administered ^{14}C-VCD

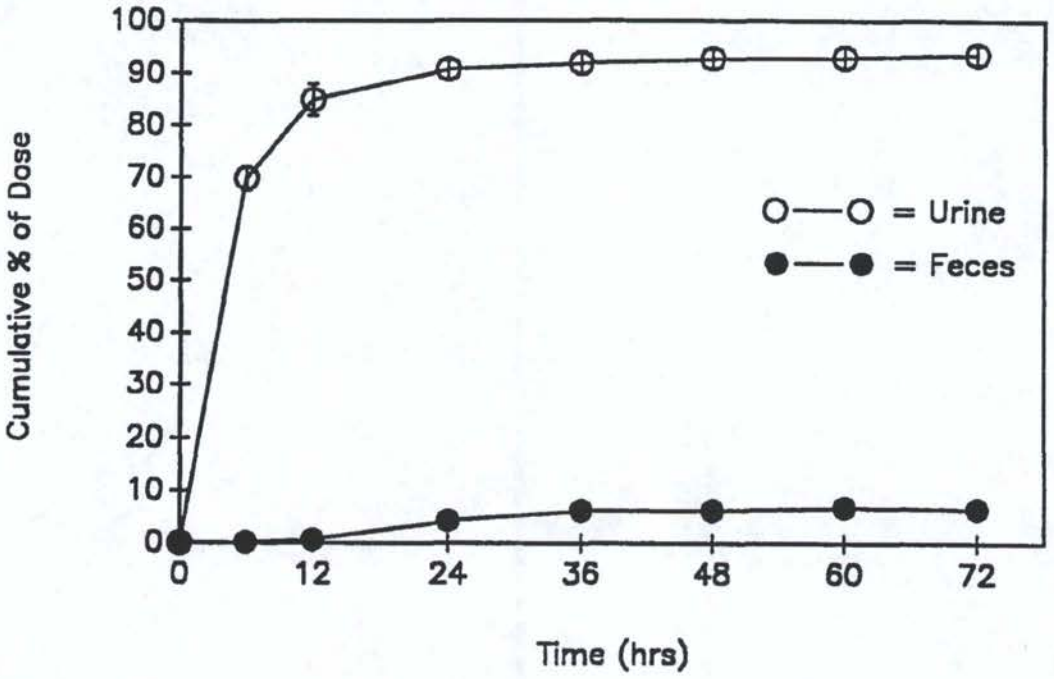

Figure 2.3 Cumulative excretion of radiolabel in urine and feces following a single intraperitoneal injection of ^{14}C-VCD (100 mg/kg; 5.5 µCi). Data represents the mean ± SD, (n=4).

Excretion of Radiolabel in Urine and Feces of Female B6C3F$_1$ Mice Following Administration of ^{14}C-VCD

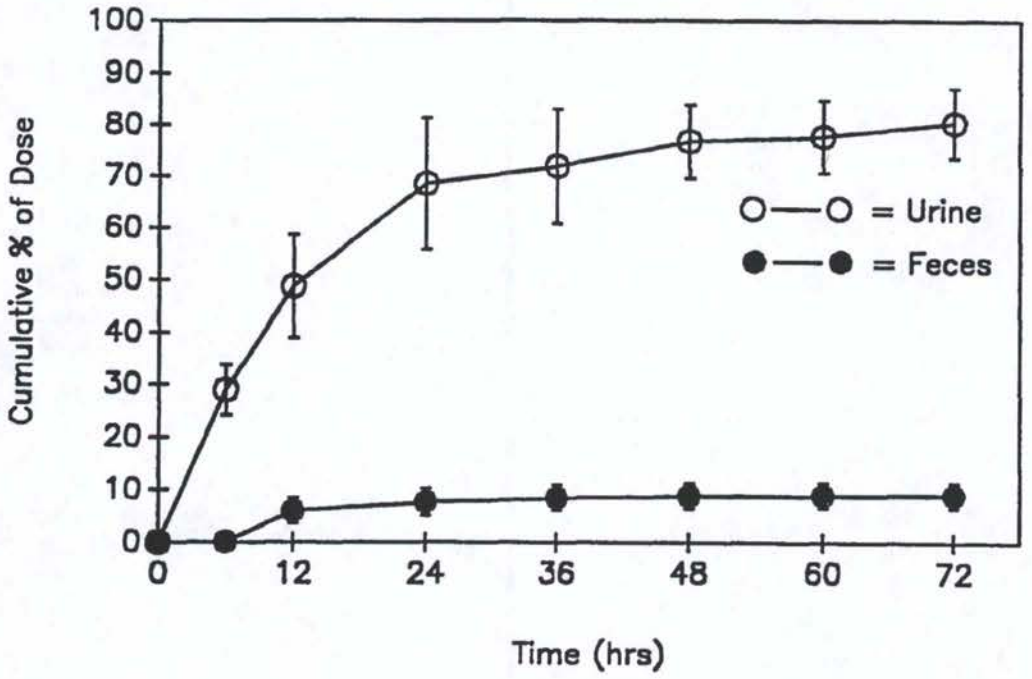

Figure 2.2 Cumulative excretion of radiolabel in urine and feces following a single intraperitoneal injection of ^{14}C-VCD (100 mg/kg; 10 µCi). Data represents the mean ± SD, (n=4).

Tissue Distribution of VCD and Its Metabolites in Female B6C3F1 Mice

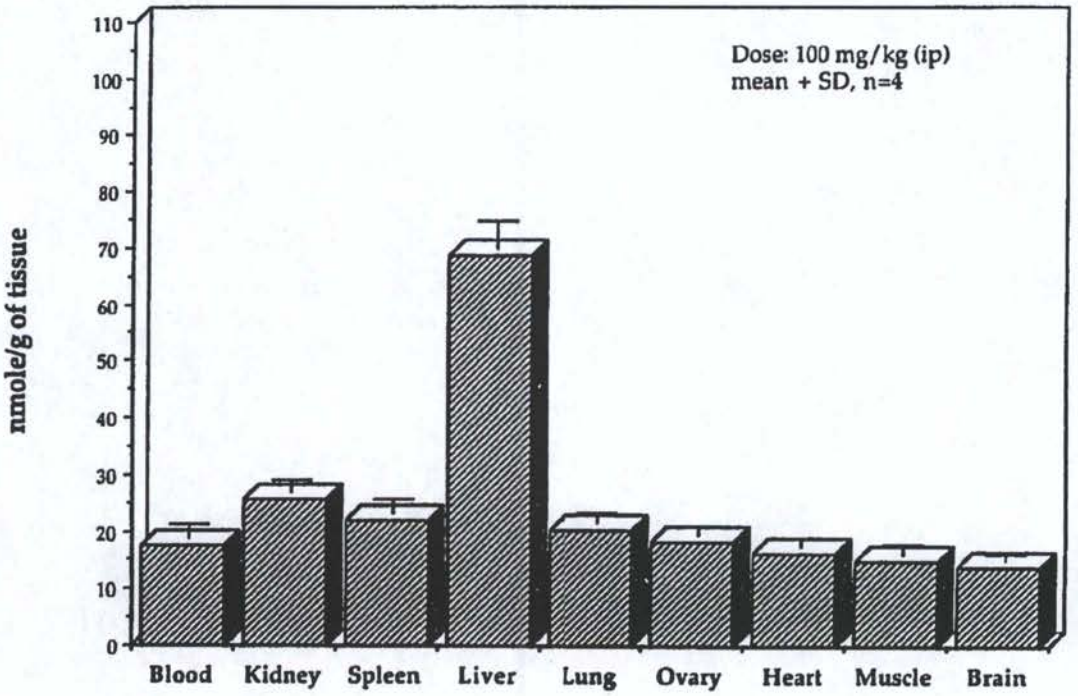

Figure 2.4 Tissue distribution of radiolabel at 72 hr following a intraperitoneal dose of ^{14}C-VCD (100 mg/kg; 5.5 µCi/mouse). Data are expressed as the mean of nmol/g of tissue ± SD, (n=4).

Tissue Distribution of VCD and Its Metabolites in Female F-344 Rats

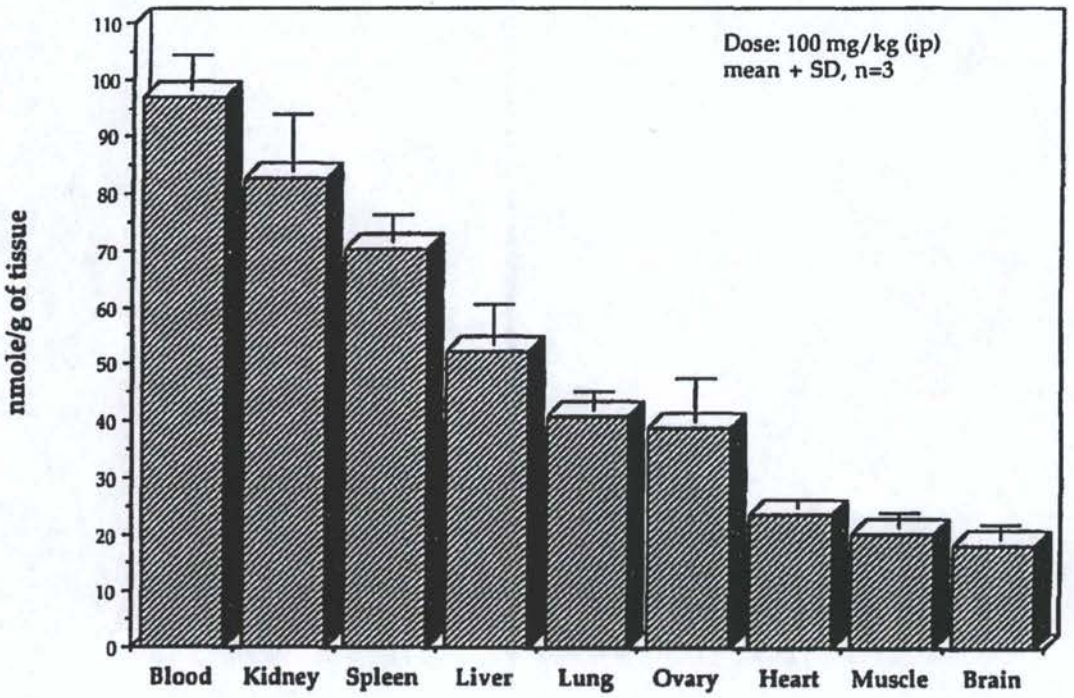

Figure 2.5 Tissue distribution of radiolabel at 72 hr following a intraperitoneal dose of ^{14}C-VCD (100 mg/kg; 10 µCi/rat). Data are expressed as the mean of nmol/g of tissue ± SD, (n=4).

concentration of the ovary was approximately one in the mouse, whereas, in the rat it was less than 0.5.

Identification of Urinary Metabolites

Normal-phase HPLC conditions were developed to separate VCD from its metabolites (Figure 2.6). Less than 1% of the administered dose was excreted in the urine as the parent compound in both species (Figure 2.7). In the rat, 4-(1,2-dihydroxy) ethyl 1,2-dihydroxycyclohexane (tetrol) was the major urinary metabolite and accounted for 60% of the administered radiolabel. The tetrol was identified by cochromatography with an authentic standard and by HPLC-mass spectrophotometry analysis (Figure 2.8, and 2.9). Additional peaks of radiolabel were also detected which were more polar than the tetrol and probably represent conjugates of ^{14}C-VCD. Incubation of urine samples with ß-glucuronidase or sulfatase did not alter the radiochromatogram profile of the urine, indicating that these enzymes failed to hydrolyze the polar peaks.

In contrast to the rat, the majority of the radioactivity excreted in mouse urine was present as very polar metabolites of ^{14}C-VCD. These probable conjugates were not hydrolyzed by ß-glucuronidase or sulfatase enzyme treatment, which suggests they are not glucuronide or sulfate conjugates. The tetrol was present in mouse urine as a minor metabolite (5-10%). It was identified by cochromatography with an authentic standard.

Radiochromatograms of Purified ^{14}C-VCD and the ^{14}C-Tetrol Standard

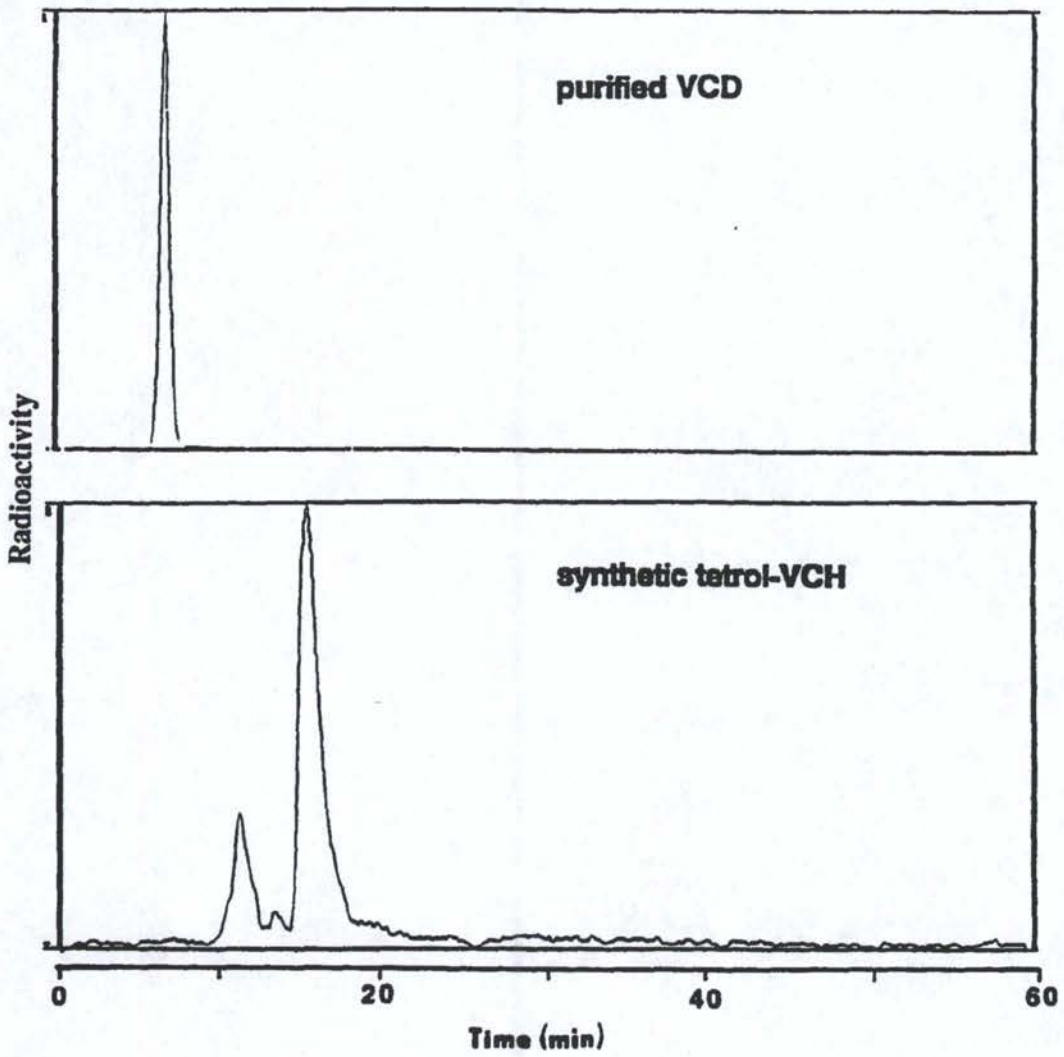

Figure 2.6 Radiochromatograms of purified ^{14}C-VCD and the ^{14}C-tetrol standard by normal-phase HPLC. Chromatography conditions are described in methods.

Radiochromatograms of Urine from Female Fischer 344 Rats and B6C3F$_1$ Mice Dosed with ^{14}C-VCD

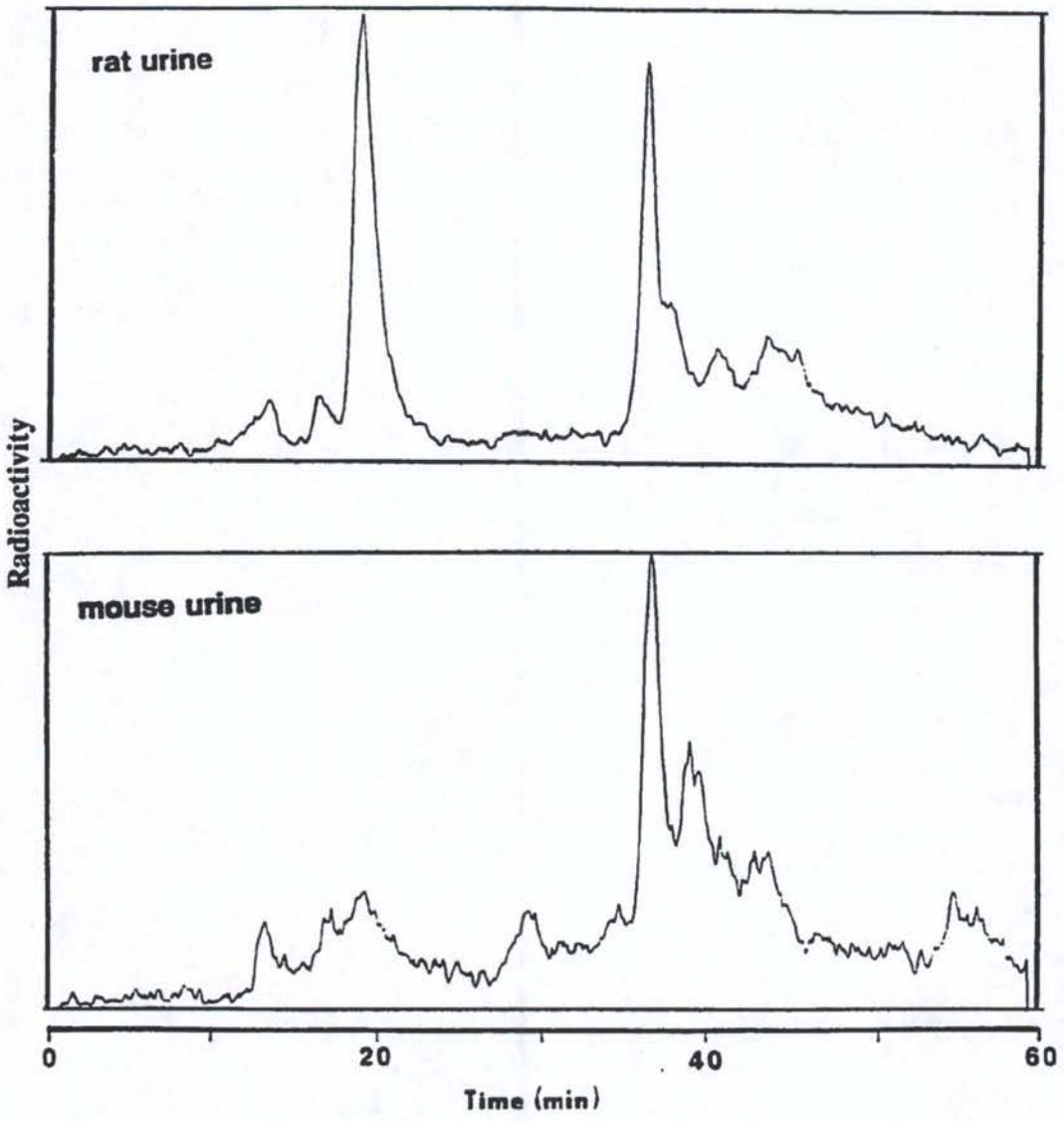

Figure 2.7 Radiochromatograms of urine from female Fischer 344 rats and B6C3F$_1$ mice dosed with ^{14}C-VCD (100 mg/kg; i.p.). Chromatography conditions are described in methods.

Mass Spectra of Synthetic 4-(1,2-dihydroxy) ethyl 1,2-dihydroxy cyclohexane

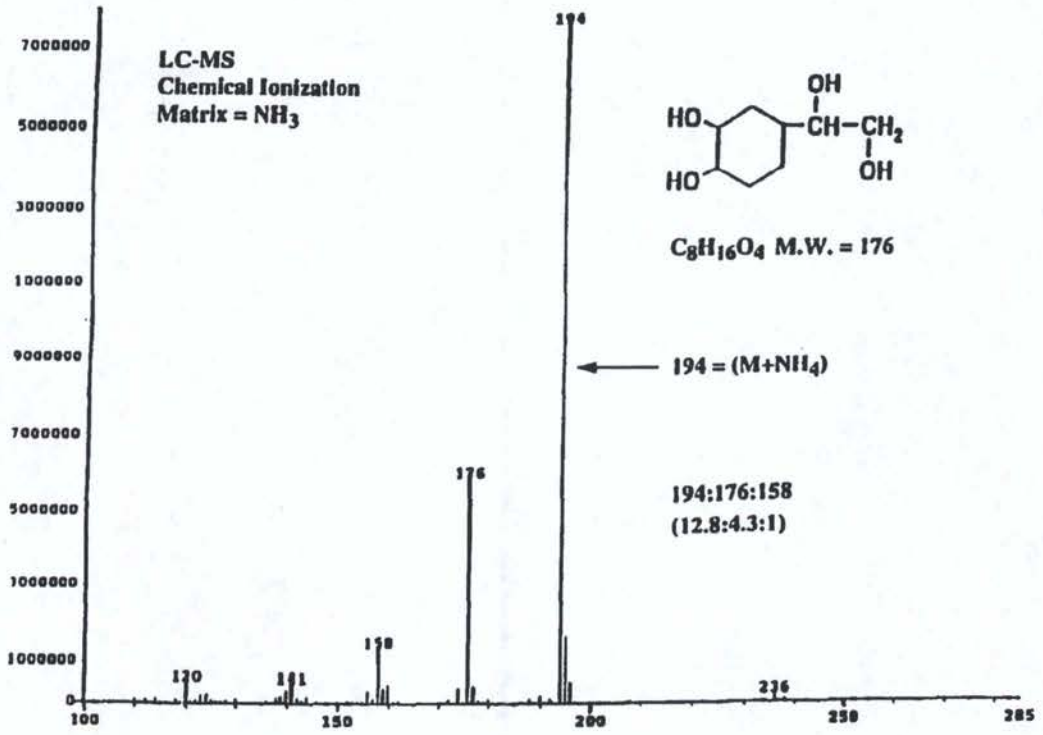

Figure 2.8 Mass spectra of synthetic 4-(1,2-dihydroxy)ethyl 1,2-dihydroxy cyclohexane. The compound was synthesized from VCD and used as a standard for LC-MS. The mass spectrometer was equipped with a thermal pneumatic nebulizer coupled with a momentum separator. The instrument was operated in the full-scan positive ion chemical ionization mode, using ammonia as the reagent gas.

Mass Spectra of 4-(1,2-dihydroxy)ethyl 1,2-dihydroxy cyclohexane in Urine from Rats Following ^{14}C-VCD Administration

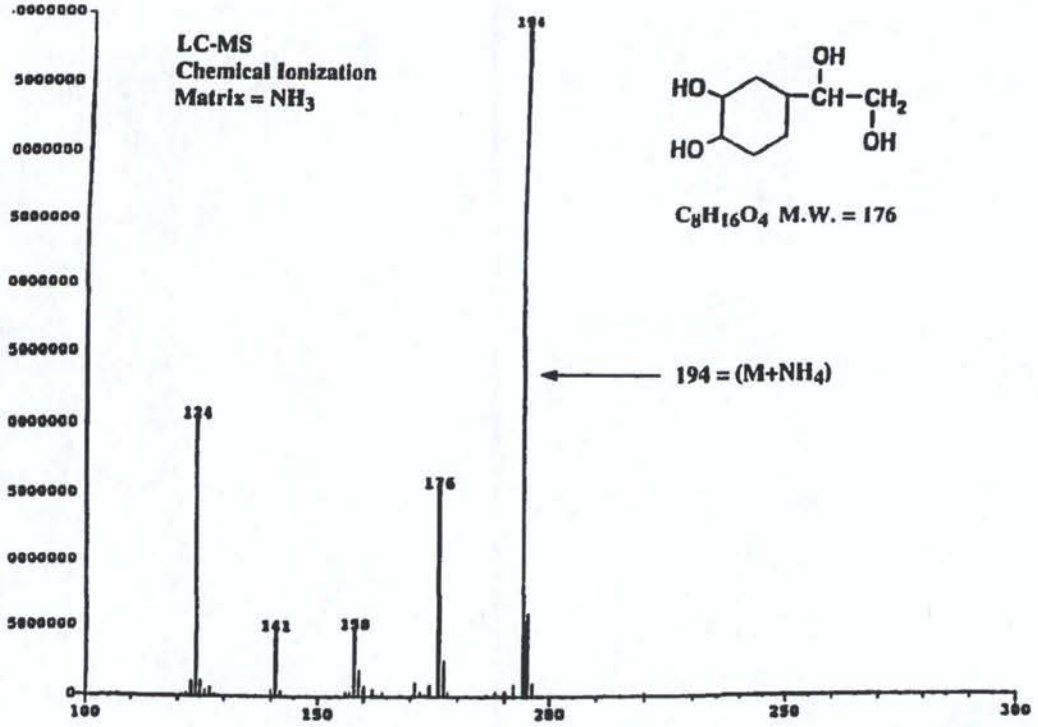

Figure 2.9 Mass spectra of 4-(1,2-dihydroxy)ethyl 1,2-dihydroxy cyclohexane in urine from a rat administered ^{14}C-VCD (100 mg/kg; 10 μCi/rat). The mass spectrometer was equipped with a thermal pneumatic nebulizer coupled with a momentum separator. The instrument was operated in the full-scan positive ion chemical ionization mode, using ammonia as the reagent gas.

DISCUSSION

Previous studies have shown that female $B6C3F_1$ mice are more susceptible to VCD-induced ovarian toxicity as compared to female Fischer 344 rats (NTP, 1989; Chhabra et al., 1990a; Chhabra et al., 1990b; and Smith et al., 1990b). To investigate species differences in the disposition and metabolism of VCD, a single dose of ^{14}C-VCD (100 mg/kg; i.p.) was administered to female Fischer 344 rats and $B6C3F_1$ mice. The dose was selected based on previous studies which have shown that repeated administration of VCD can cause cancer at the site of application and ovarian toxicity (NTP, 1989; and Smith et al., 1990b).

The results indicate that VCD was rapidly metabolized and excreted predominantly in the urine of both species. However, the excretion rate was higher in the rat compared to the mouse. Since only metabolites of VCD are excreted in the urine, the results indicate that the rat metabolizes VCD to polar metabolites more rapidly than the mouse. The difference in the urinary profile of metabolites indicates different pathways of metabolism of VCD between the two species. The rat appears to use two enzyme systems to metabolize VCD, while the mouse primarily uses one. In the rat, VCD is preferentially hydrolyzed by epoxide hydrolase to form the tetrol, or VCD can be conjugated. Whereas, the mouse primarily conjugates the epoxides of VCD. It appears the rat epoxide hydrolase has higher activity toward VCD when compared to the mouse. A previous study examined the microsomal epoxide hydrolase activity toward a structurally similar compound, styrene oxide, and found much higher activity in the rat compared to the mouse (Ota and Hammock, 1980). The reduced capacity of the female mouse to metabolize VCD by epoxide hydrolase may render the mouse more susceptible to VCD-induced ovarian toxicity compared to the rat.

The results of this study collaborate earlier findings where Sipes et al., (1989) investigated the disposition of a single dermal application of ^{14}C-VCD in female Fischer 344 rats and B6C3F$_1$ mice. Results from both studies indicate tha major route of elimination of radiolabel was via the urine in both species (Sipes et al., 1989). Further, results from both studies indicate less than 5% of the dose was excreted in the feces of both species. In the earlier study, the tissue:blood ratio of radiolabel in mouse ovarian tissue was five-fold greater compared to rat ovarian tissue. However, in this study, the ovarian tissue:blood concentration was approximately one in the mouse, whereas, in the rat it was less than 0.5. The difference in tissue to blood ratios observed between the studies may be related to different routes of administration (i.p. versus dermal) and the time when tissues were collected, 24 hr after dermal application versus 72 hr following intraperitoneal administration..

Identification of one of the urinary metabolites of VCD, the tetrol, was accomplished by cochromatography with an authentic standard and by HPLC-mass spectrophotometry. A previous study also identified this metabolite from rabbit liver microsomal incubations (Watabe and Sawahata, 1976). Also present as urinary metabolites of VCD were at least two very polar unidentified metabolites/conjugates. Treatment of urine with enzymes that hydrolyze glucuronide (ß-glucuronidase) or sulfate (sulfatase) conjugates, did not cleave the polar metabolites. No change in the radiochromatogram was observed. These metabolites may represent conjugates with GSH. Giannarini et al. (1981) reported depletion of liver GSH following a single dose of VCD. Further, styrene oxide, cyclohexene oxide, (Hayakawa et al., 1975), and 3,4-epoxyvinlycyclohexe (Boyland and Williams, 1965), which are structurally similar to

VCD, are all good substrates for glutathione-S-transferases. Studies are underway to identify these polar metabolites.

Previous studies which have shown VCD to be carcinogenic and ovotoxic all used chronic exposure of VCD (NTP, 1989; Chhabra et al., 1990b; and Smith et al., 1990b). The results suggests the mouse has a reduced capacity of epoxide hydrolase activity and is therefore dependent on the conjugation pathway for metabolism and elimination of VCD. However, chronic exposure of VCD could deplete the cosubstrate necessary for the conjugation and excretion of VCD, thereby, rendering that species more susceptible to VCD-induced toxicity. Results from the treatment of urine with enzymes which hydrolyze ß-glucuronides and sulfate conjugates implicate the polar urinary metabolites as VCD-GSH conjugates. In subsequent chapters, further evidence will be indicated which will support that these polar metabolites are VCD-GSH conjugates.

In summary, this study was undertaken to determine if a species difference exists in the disposition and/or metabolism of VCD between female Fischer 344 rats and B6C3F_1 mice. A difference in the disposition and metabolism of VCD was observed between rats and mice. The rat eliminated radiolabel more rapidly than the mouse. Also a difference in the urinary profile of VCD metabolites suggests different pathways of metabolism of VCD between the two species. The rat uses two enzyme pathways to metabolize VCD, whereas, the mouse primarily uses one. These differences in the disposition and metabolism may in part, explain why the mouse is more susceptible to VCD-induced toxicity.

CHAPTER 3

TOXICOKINETICS AND BILIARY EXCRETION OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE IN FEMALE FISCHER 344 RATS AND B6C3F$_1$ MICE

INTRODUCTION

4-Vinyl-1-cyclohexene diepoxide (VCD) is a small molecule with a molecular weight of 140.2. It is manufactured by epoxidation of 4-vinylcyclohexene with peroxyacetic acid (Wallace, 1964). VCD has been used as a chemical intermediate in condensation reactions with dicarboxylic acids and as a monomer for preparation of polyglycols containing nonreactive epoxy groups (IARC 1976). Occupational exposure to VCD occurs primarily via inhalation during the manufacture of polyesters and plastic coatings (Miller, 1978). In the mid-1980's, the National Occupational Exposure Survey estimated that 70,000 workers in the United States are potentially exposed to VCD (NIOSH, 1988). Because of the potential for human exposure, a threshold limit value/time-weighted average of 10 ppm (60 mg/m3) for skin has been recommended by the American Conference of Governmental Industrial Hygienists (ACGIH, 1986).

Many studies have demonstrated the mutagenic activity of VCD in *Salmonella typhimurium*,with or without S9 metabolic activation, particularly with base-substitution indicator strains TA100 and TA1535 (Murray and Cummins, 1979; Wade et al., 1979; Frantz and Sinsheimer, 1981; Mortelmans et al. 1986; and NTP, 1989). Turchi et al. (1981) showed that VCD induced point mutations and chromosomal abnormalities (anaphase bridges and micronuclei) in V79 Chinese hamster lung cells. Further, VCD caused gene reversions, conversions, and mitotic crossing-over in *Saccharomyces cervisiae* (Bronzetti et al., 1980). Collectively, these studies indicate that VCD is a direct acting mutagen.

Despite the positive genotoxicity data and the potential for human exposure, there are limited studies describing the metabolism and disposition of VCD. Watabe and Sawahata (1976) investigated the *in vitro* metabolism of VCD in rabbit liver microsomes.

Hydrolysis of one or both of the epoxides of VCD was observed, resulting in the formation of corresponding diols (Watabe and Sawahata, 1976). The NTP conducted dermal application studies on male and female F-344 rats and B6C3F_1 mice over a 2 yr period (NTP, 1989). VCD caused squamous and basal cell carcinomas of the skin in males and females of both species. Further, following dermal application, VCD was ovotoxic to mice, but not rats, as indicated by ovarian atrophy, decreased number of follicles, and ovarian neoplasms (NTP, 1989; and Chhabra et al. 1990a and 1990b). However, following repeated intraperitoneal dosing of rats and mice with VCD, ovarian toxicity was observed in both species. However, the mouse was more sensitive to VCD than the rat.

In the preceding chapter the disposition and metabolism of a single intraperitoneal injection of ^{14}C-VCD was compared in female Fischer 344 rats and B6C3F_1 mice. The results indicate that majority of the dose was excreted in the urine by both species. However, the radiolabel was excreted more rapidly in the rat compared to the mouse. For example, at 6 hr the rat excreted approximately 70% of the radiolabel in the urine compared to 30% in the mouse. Also, the urinary metabolite profile was different between the two species. In the rat, the tetrol was the major urinary metabolite, with small amounts of other polar metabolites. In contrast, the tetrol was only a minor metabolite in B6C3F_1 mice, and the major metabolites appeared to be polar conjugates of VCD. The differences in the disposition and metabolism of VCD may in part explain why the mouse is more susceptible to VCD-induced ovotoxicity than the rat (Smith et al., 1990b; Chhabra et al., 1990a and 1990b). To further characterize the disposition and metabolism of VCD in female Fischer 344 rats and B6C3F_1 mice the toxicokinetic parameters of a single intravenous dose of ^{14}C-VCD was investigated. Also, to further our understanding of the elimination process, the biliary excretion of ^{14}C-VCD and its

metabolites was determined. A species difference in these parameters could explain, in part, the species difference in susceptibility to VCD-induced ovarian toxicity.

MATERIALS AND METHODS

Animals

Adult female B6C3F$_1$ mice (19-22g) and female Fischer 344 rats (150-170g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Rats were housed in hanging wire cages and mice in polycarbonate plastic pans with hardwood chip bedding. The animals were allowed free access to food (Teklad 4% mouse/rat; Harlan Teklad, Madison, WI) and water. The animals were maintained on a 12 hr light/dark cycle, at an ambient room temperture of 21-22°C. The animals were acclimated for at least 7 days prior to use. For the toxicokinetic study, jugular vein cannulated female Fischer 344 rats (150-170g) were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA). The rats were individually housed in metabolism cages in the same environment as above for at least 2 days prior to use. The jugular cannula was kept free of blood clots by flushing the cannula daily with saline and filling with heparin-saline (1:1) solution.

Chemicals

4-vinylcyclohexene diepoxide (VCD) and urethane were purchased from Aldrich Chemical Co. (Milwaukee, WI). n-Ethylmaleimide, phenolphthalein glucuronide, p-nitrophenyl sulfate, lyophilized ß-glucuronidase type VII-A (from *Escherichia coli*), and sulfatase type V (from limpets) were purchased from Sigma Chemical Co. (St. Louis,

MO). L-Buthionine-S-R-sulfoximine was obtained from ICN Biochemicals (Cleveland, OH). γ-Glutamyl glutamate was purchased from Vega Biochemicals (Tucson, AZ). CarboSorb^{R} and FLO SCINT™ II cocktail were obtained from Packard Instruments (Chicago, IL). Universol™ Cocktail was purchased from ICN Radiochemicals (Irvine, CA). PE-50 polyethylene tubing was purchased from Clay Adams Co. (Parsippany, NJ). All other chemicals and chromatography solvents used were either analytical grade or HPLC grade.

Chromatography

^{14}C-VCD was purified using a 10 μm Phenomenex Partisil^{R} silica semi-preparative column (250 x 10 mm). The purification procedure and HPLC conditions are as described in methods (Chapter 2). Chemical purity of unlabeled and ^{14}C-VCD was quantified using a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA) fitted with a 0.32 mm x 10 M RSL-300 capillary column (Alltech Associates; Deerfield, IL) and a flame ionization detector (FID). Other gas chromatography conditions and procedure are described in methods (Chapter 2).

VCD and its metabolites were separated and quantified from plasma and bile using a 7 μm Phenomenex CN Zorbax^{R} HPLC column (4.6 x 250 mm). The mobile phase consisted of 90:10 hexane:isopropanol at time zero, a gradient to 50:50 hexane:isopropanol at 20 min, and a further gradient to 100% methanol at 60 min. Radioactivity was analyzed using a flow scintillation detector (Radiomatic Instruments, Meriden, CT), with a combined flow rate of 3 ml/min (2:1 ratio of FLO SCINT™ II cocktail:mobile phase).

Biliary Excretion Study

The biliary excretion study was conducted in female Fischer 344 rats. Rats were anesthetized with urethane (1.5 g/kg, i.p.) and bile duct and right jugular vein were cannulated with PE-50 polyethylene tubing. Throughout the experiment saline (1.5 ml/hr) was infused via the jugular vein. Also, the core body temperature of the rats was maintained at 36 °C ± 0.5 °C with an electric heating pad. After a steady bile flow was established (about 15 min), a bolus dose of ^{14}C-VCD (10 mg/kg, 10 uCi) was administered via the jugular vein and bile samples collected at 30 min intervals for 6 hr. At 6 hr, the rats were killed by carbon dioxide inhalation and necropsy performed. Triplicate tissue aliquots (100-200 mg) were combusted to $^{14}CO_2$ using a Packard Model 306 Sample Oxidizer (United Technologies; Downers Grove, IL), and analyzed for radioactivity using a Beckman (LS 5000 TDC) scintillation counter. Aliquots of bile samples (100 µl) were added to 6.0 ml scintillation fluid and analyzed for total radioactivity using a scintillation counter. Bile samples were also analyzed for parent compound and its metabolites using HPLC as described.

GSH Depletion Study

Hepatic GSH was depleted using a modified procedure of Griffith and Meister (1979). Rats received a total dose of 2 g/kg L-buthionine-S,R-sulfoximine (BSO) in distilled water. BSO (1 g/kg; i.p.) was administered twice at 4.5 and 3 hr before administration of VCD. ^{14}C-VCD (10 mg/kg; 10 µCi) was administered 3 hr after the last dose of BSO. Bile was collected as described for the biliary excretion study. At 6 hr, the rats were killed by carbon dioxide inhalation and partial necropsy performed. Aliquots of bile samples were analyzed for total radioactivity using a scintillation

counter. Bile samples were also analyzed for parent compound and its metabolites using HPLC.

Determination of Hepatic GSH and GSSG Content

Liver homogenates were prepared (20% liver) in distilled water. Oxidized and reduced GSH were quantified by the method of Fariss and Reed (1987). This method is based on the formation of S-ethyl derivatives of free thiols by n-ethylmaleimide followed by derivatization of primary amines with Sanger's reagent, 1-fluoro-2,4-dinitrobenzene. Following the derivatization procedure, nanomole levels of sulfur-containing amino acids are detected at 365 nm. GSH and GSSG were separated using reverse-phase ion-exchange HPLC. The analytical column was a 5 µm Alltech spherisorb amino propyl column (4.6 x 150 mm). The mobile phase consisted of (4:1) methanol:water with 1% glacial acetic acid followed by a gradient to 100% of 0.5 M sodium acetate buffer in 64% methanol at 15 min. γ-Glutamyl glutamate was used as an internal standard. With these HPLC conditions, GSH elutes at 11.2 min and GSSG elutes at 22.1 min.

Enzyme Assay

Bile from female Fischer 344 rats dosed with ^{14}C-VCD (10 mg/kg; 10 µCi) was incubated with 800 units ß-glucuronidase or 25 units sulfatase. The assay procedure and conditions were those described in the Sigma insert which accompanies enzymes. Standard curves using phenolphthalein glucuronide and p-nitrophenyl sulfate were used to determine enzyme activity.

Toxicokinetic Study

A bolus dose of ^{14}C-VCD (10 mg/kg; 8.75 µCi) was administered intravenously

to female Fischer 344 rats via the jugular cannula. Blood samples (300 µl) were collected at selected times (1, 3, 5, 10, 30, 60, 120, 240, and 360 min). At each time point, an equal volume of saline was infused to replace the blood volume from sample collection. At 6 hr, the rats were killed by CO_2 inhalation and partial necropsy performed.

To study the toxicokinetics of VCD in female mice, a bolus dose of ^{14}C-VCD (10 mg/kg; 6.0 µCi) was administered via the tail vein. At selected times (1, 3, 5, 10, 30, 60, 120, 240, and 360 min), mice were killed by CO_2 inhalation and blood samples collected from the inferior vena cava. A partial necropsy was performed on all mice at each time point. Aliquots of blood and tissues from both rats and mice (100-200 mg) were combusted to $^{14}CO_2$ using a Packard Model 306 Sample Oxidizer (United Technologies; Downers Grove, IL), and analyzed for radioactivity as described for the biliary excretion study. Plasma samples from each species were also analyzed for ^{14}C-VCD and its metabolites using HPLC.

Synthesis of GSH-^{14}C-VCD Conjugate

GSH conjugates of VCD were synthesized from VCD and reduced GSH. The reaction contained 1 mM VCD and 5 mM GSH in 10 mM Tris buffer (pH 9.0) at room temperature. ^{14}VCD (1 µCi/ml of reaction) was included in the reaction mixture to assist in HPLC separation. The reaction mixture was stirred continuously and the pH was maintained at 9.0. An aliquot of the reaction mixture was analyzed by HPLC every 2 hr to determine progress of the reaction. HPLC separation of the reaction mixture indicated that the parent compound was either conjugated with GSH or the epoxides moieties were hydrated to form the corresponding diols.

The ^{14}C-VCD-GSH conjugate was purified using a 10 μm Phenomenex Partisil^{R} silica semi-preparative column (250 x 10 mm). The mobile-phase was an isocratic solution of hexane:isopropanol (98:2), at 4 ml/min for 30 min. Radioactivity was analyzed by a flow scintillation detector (Radiomatic Istruments, Meriden, CT), at a combined flow rate of 8 ml/min (1:1 ratio of FLO SCINT™ II cocktail:mobile phase). Appropriate fractions containing ^{14}C-VCD-GSH conjugate were directly collected from the HPLC. The ^{14}C-VCD-GSH fractions were pooled and the solvents removed by a Speed-Vac (geneVac, model SF 50).

The normal-phase purified ^{14}C-VCD-GSH conjugate still contained bile acids and other bile constituents which co-elute with ^{14}C-VCD-GSH conjugates. Therefore, these ^{14}C-VCD-GSH conjugates were purified again using a 10 μm Econosil C-18 reverse-phase semi-preparative column (250 x 10 mm) at 3 ml/min. The mobile-phase was 1% glacial acetic acid (pH=3.5) and methanol (90:10) at time zero followed by a gradient to 100% methanol at 60 min. The absorbance of the eluate was monitored at 260 nm. Fractions (1.5 ml) were collected directly from the HPLC column and aliquots of each fraction were analyzed for radioactivity using a Beckman (LS 5000 TDC) scintillation counter. Fractions which contained ^{14}C-VCD-GSH conjugates were pooled and aqueous solvents removed by a Flexi-Dry lypholizer (FTS^{R} Systems, Inc., Stone Ridge, N.Y.).

Calculation of Toxicokinetic Parameters

The first-order elimination rate constant (K) was determined by nonlinear regression using the least squares method. The area under the plasma concentration-time curve (AUC) was determined by the trapezoidal rule. Other equations used include:

Plasma half-life or $t_{1/2}$ = 0.693 / K;

Mean residence time or $MRT_{i.v.}$ = 1 / K;

Systemic clearance or CLs = $dose_{i.v.}$ (g/kg) / AUC;

Volume of distribution or Vd = $dose_{i.v}$ (g/kg) / blood conc. (g/L).

RESULTS

Toxicokinetic Study

To characterize the elimination of VCD in female Fischer 344 rats and $B6C3F_1$ mice, the toxicokinetic properties of VCD were investigated. A single dose of ^{14}C-VCD (10 mg/kg) was administered to rats (8.75 μCi) via the jugular cannula and mice (6.0 μCi) via the tail vein. Aliquots of plasma from each time point were analyzed by HPLC. VCD was rapidly eliminated from the plasma in both species (Figures 3.1 and 3.2). The mean residence time (MRT) in plasma for VCD was approximately 15 min, and 5 min, in the rat and mouse, respectively. In contrast, the tetrol metabolite had a much longer MRT in both the species. The volume of distibution (Vd) for VCD was five fold greater in the mouse than that obtained in the rat. The systemic clearance (CLs) for VCD was more rapid in the mouse (32.7 L/kg·hr) compared to the rat (2.0 L/kg·hr). Other toxicokinetic parameters obtained for VCD and tetrol metabolite are described in Table 3.1.

The distribution of radiolabel was examined in selected tissues from $B6C3F_1$ mice following a single intravenous administration of ^{14}C-VCD (10 mg/kg; 6 μCi). The tissue:blood ratio of mouse liver, ovary, and adrenal gland at each time point are reported

Plasma Profile Following Intravenous Dose of ^{14}C-VCD from Female Fischer 344 Rats

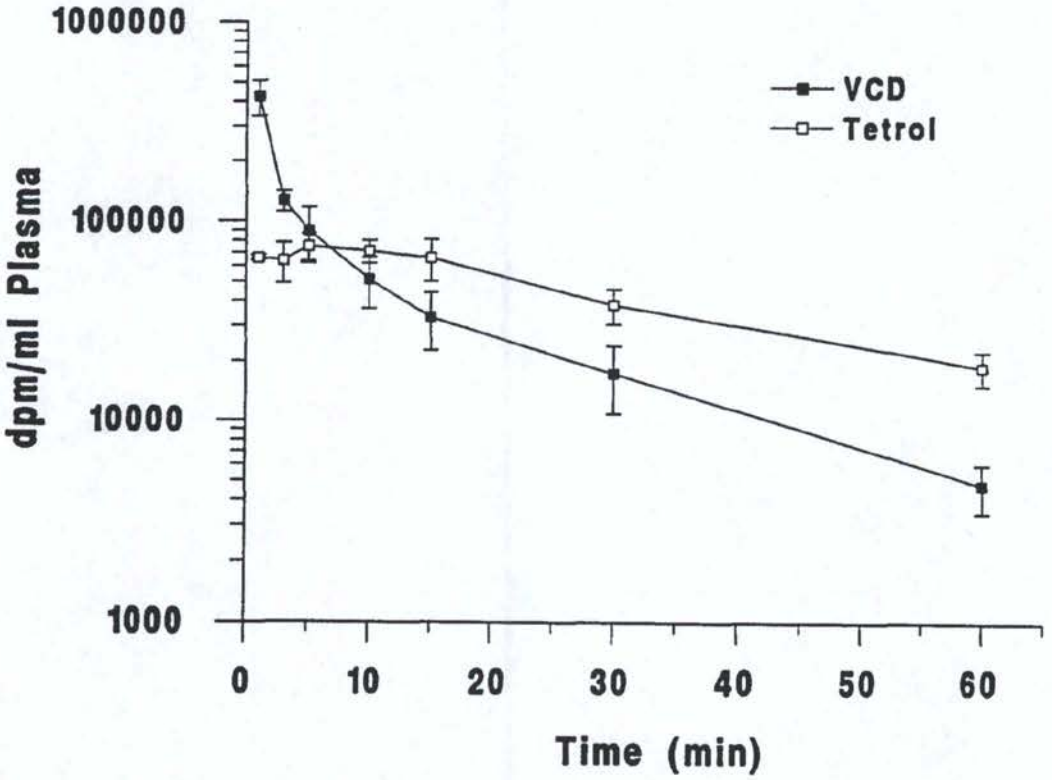

Figure 3.1 Plasma profile of VCD and tetrol metabolite from female Fischer 344 rats following intravenous administration of ^{14}C-VCD (10 mg/kg; 8.75 μCi). Blood was collected at selected times over 6 hr and plasma analyzed by HPLC. Data represent the mean of 6 rats ± S.D.

Plasma Profile Following Intravenous Dose of ^{14}C-VCD from Female B6C3F$_1$ Mice

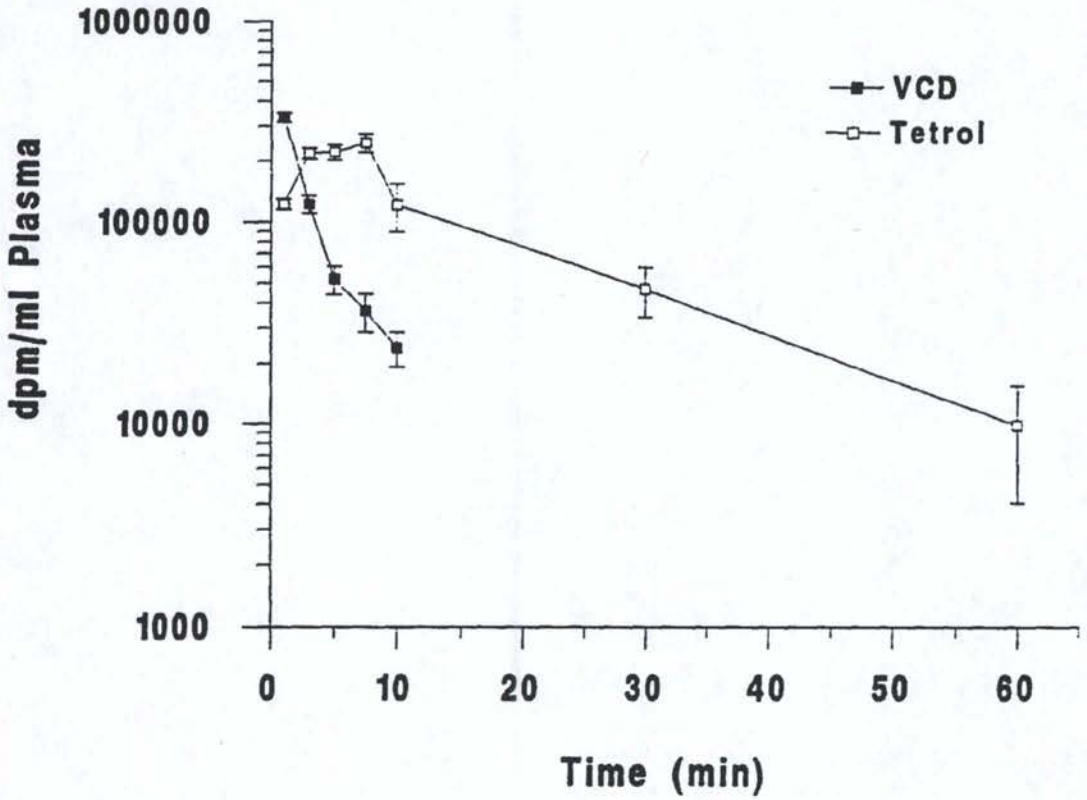

Figure 3.2 Plasma profile of VCD and tetrol metabolite from female B6C3F$_1$ mice following intravenous administration of ^{14}C-VCD (10 mg/kg; 6.0 µCi). Mice were killed at selected times over 6 hr, blood removed, and plasma analyzed by HPLC. Data represent the mean of 4 mice ± S.D.

Toxicokinetic Parameters of VCD and Tetrol from Female Fischer 344 Rats and B6C3F$_1$ Mice Following an Intravenous Dose of ^{14}C-VCD

	$t_{1/2}$ (min)	K (min^{-1})	AUC (uM·hr)	CLs (L/kg·hr)	VD (L/kg)	MRT (min)
Mice						
VCD	4.4	0.16	1.43	32.7	208.7	4.7
Tetrol	24.1	0.03	12.37	N.A.	N.A.	30.6
Rats						
VCD	14.2	0.05	2.72	2.0	40.1	15.0
Tetrol	19.9	0.04	3.98	N.A.	N.A.	41.7

Table 3.1 Toxicokinetic parameters of VCD and Tetrol were determined from female Fischer 344 rats and B6C3F$_1$ mice administered an intravenous dose of ^{14}C-VCD (10 mg/kg; 8.75 μCi). Blood was collected over 6 hr and plasma analyzed by HPLC. Each value was determined from the mean of 4 animals ± S.D. N.A.=not applicable.

in table 3.2. The tissue:blood ratio for ovary was one at 1 min and increased to 3.8 and 3.4 at 2 hr and 4 hr following the dose, respectively. The study protocol for rats was not designed to yield the distribution of radiolabel at each time point, therefore, a direct comparison between the species is not available.

Identification of Plasma Radiolabel

Aliquots of plasma from both species at each time point were analyzed by normal-phase HPLC. The tetrol was the only plasma metabolite observed throughout the study in both species. It was identified by cochromatography with an authentic standard. This metabolite was present in the plasma at the earliest time point (1 min) and was still present in the plasma at 60 min following the dose in both species (Figure 3.3).

Biliary Excretion of Radiolabel

The cumulative excretion of radiolabel in bile following a bolus dose of ^{14}C-VCD in female Fischer 344 rats is shown in Figure 3.4. Following intravenous administration of ^{14}C-VCD (10 mg/kg; 10 μCi/rat) approximately 17% of the dose was excreted in the bile in 6 hr. The excretion of radiolabeled VCD and its metabolites reached plateau about 2 hr following administration of ^{14}C-VCD. The flow of bile remained constant (0.2-0.25 ml/0.5 hr) throughout the experiment.

Following administration of BSO (2 g/kg; i.p.), hepatic GSH levels were reduced to 30% of contol levels. Intravenous administration of VCD or BSO treatment did not alter hepatic GSSG levels. The cumulative excretion over 6 hr of radiolabel in the bile following a bolus intravenous dose of ^{14}C-VCD in BSO treated female Fischer 344 rats was only 4.5% of the dose.

Distribution of Radiolabel in Selected Tissues Following an Intravenous Dose of ^{14}C-VCD from Female $B6C3F_1$ Mice

Time (min)	Liver:Blood Ratio	Ovary:Blood Ratio	Adrenal:Blood Ratio
1	0.95	1.02	1.58
3	2.21	1.68	1.47
5	2.35	1.47	1.22
10	2.38	1.51	1.11
30	2.37	2.15	2.14
60	2.72	2.56	3.02
120	4.02	3.80	3.39
240	5.01	3.41	3.50

Table 3.2 Selected tissue distribution of raiolabel from female $B6C3F_1$ mice following an intrabenous dose of ^{14}C-VCD (10 mg/kg; 6 μCi). Mice were killed at selected times over 6 hr, aliquots of blood and tissues were combusted to $^{14}CO_2$ and analyzed for radioactivity. Data are expressed as tissue:blood ratio and represent the mean of 4 mice.

HPLC Radiochromatograms of Plasma from Female F-344 Rats Administered ^{14}C-VCD

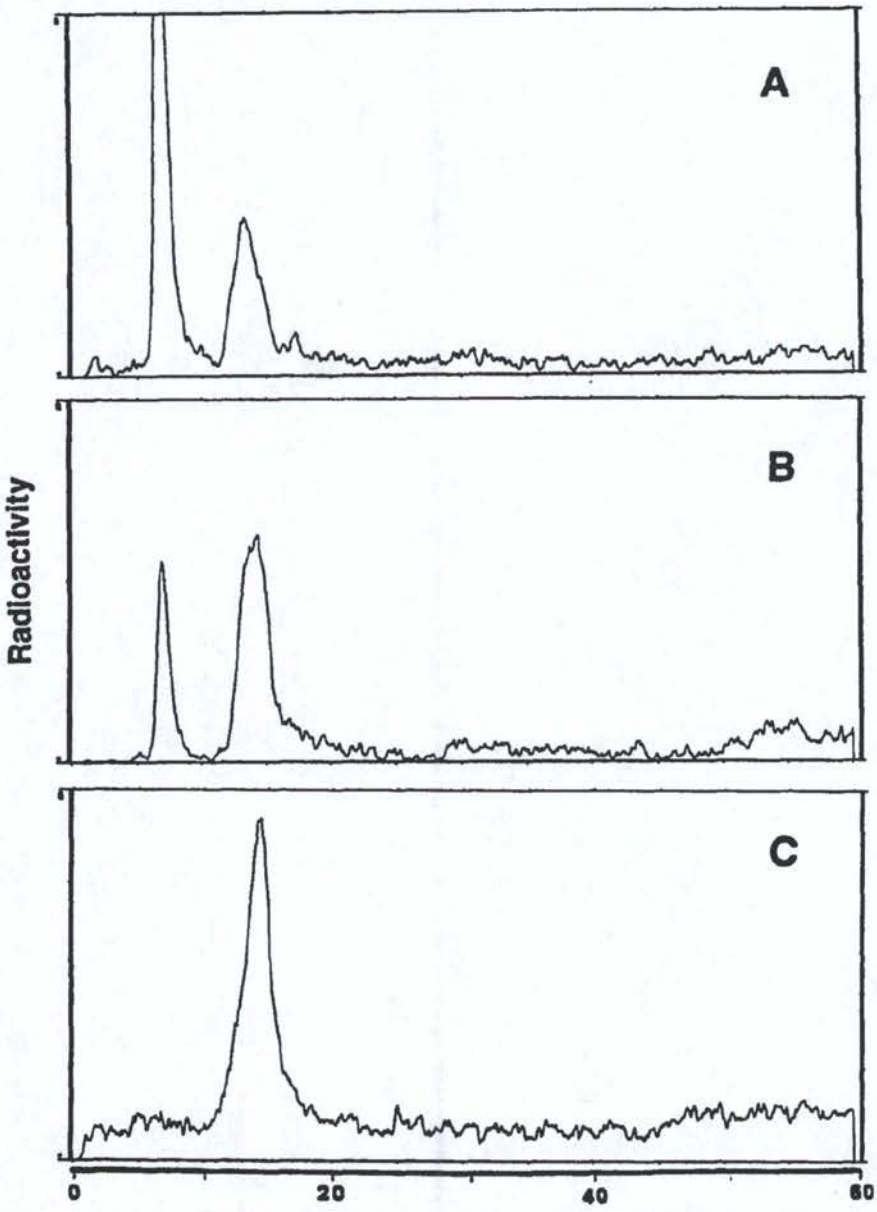

Figure 3.3 Radiochromatograms of plasma from female Fischer 344 rats following an intravenous dose of ^{14}C-VCD (10 mg/kg; 10 μCi). Radiochromatograms are plasma samples collected at: A) 1 min, B) 5 min, and C) 30 min following administration of ^{14}C-VCD. HPLC conditions are described in methods.

Biliary Excretion of Radiolabel in Female Fischer 344 Rats Administered ^{14}C-VCD

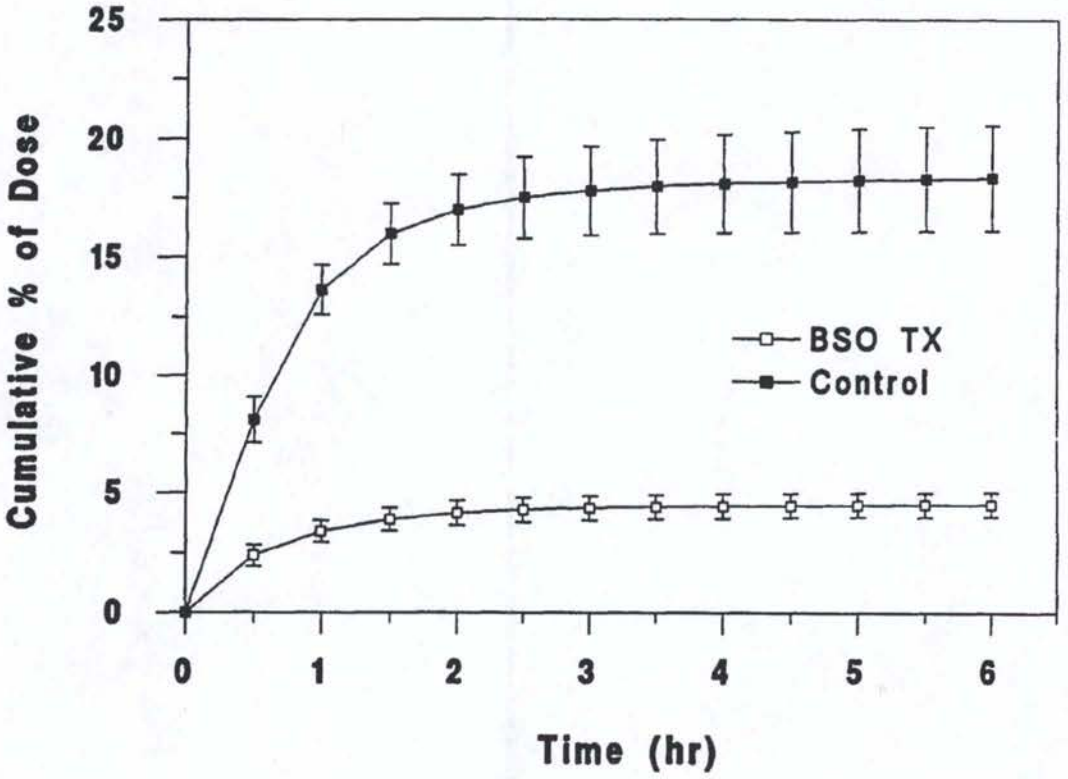

Figure 3.4 Biliary excretion of radiolabel in female Fischer 344 rats. Cummulative excretion of radiolabel in bile following a single intravenous dose of ^{14}C-VCD (10 mg/kg; 10 µCi/rat). BSO pretreated rats received a total dose of 2 g/kg BSO (i.p.). Data are expressed as mean percent of dose ± SD, (n=3 or 4).

Tissue Distribution of Radiolabel

The tissue distribution of radiolabel was examined in female Fischer 344 rats 6 hr following a single intravenous administration of ^{14}C-VCD. The highest tissue concentrations of radiolabel were found in the liver, kidney, and brain as shown in (Figure 3.5). These three tissues had a 2-3 fold higher concentration of radiolabel than the blood. The tissue:blood concentration ratio in spleen, ovary, and lung were approximately one. All other tissues examined had a tissue:blood concentration of less than one.

Identification of Biliary Metabolites

Normal-phase HPLC conditions were developed to separate VCD from its metabolites present in bile (Figure 3.6). Approximately 1% of the radiolabel present in bile was parent compound. The tetrol was a minor biliary metabolite of VCD, accounting for approximately 5% of the radiolabel present in the bile at all time points examined. The majority of radiolabel excreted in the bile was present in at least two very polar metabolites. These probably represent conjugates of ^{14}C-VCD. When bile was subjected to treatment with either ß-glucuronidase or sulfatase, the chromatographic profile of these metabolite peaks did not change. Thus, it is unlikely that these metabolites are glucuronide or sulfate conjugates of VCD metabolism.

Bile from rats pretreated with BSO, followed by single intravenous dose of ^{14}C-VCD was examined for parent compound and its metabolites using HPLC. Approximately 3% of the radiolabel was present as the parent compound (Figure 3.7), and 5-8% as the tetrol of VCD. The majority of radiolabel excreted in the bile was present in at least two very polar metabolites, possible conjugates of ^{14}C-VCD. The most polar of these two probable conjugates coeluted on HPLC with a synthetic GSH-

Tissue Distribution of VCD and Its Metabolites in Female F-344 Rats

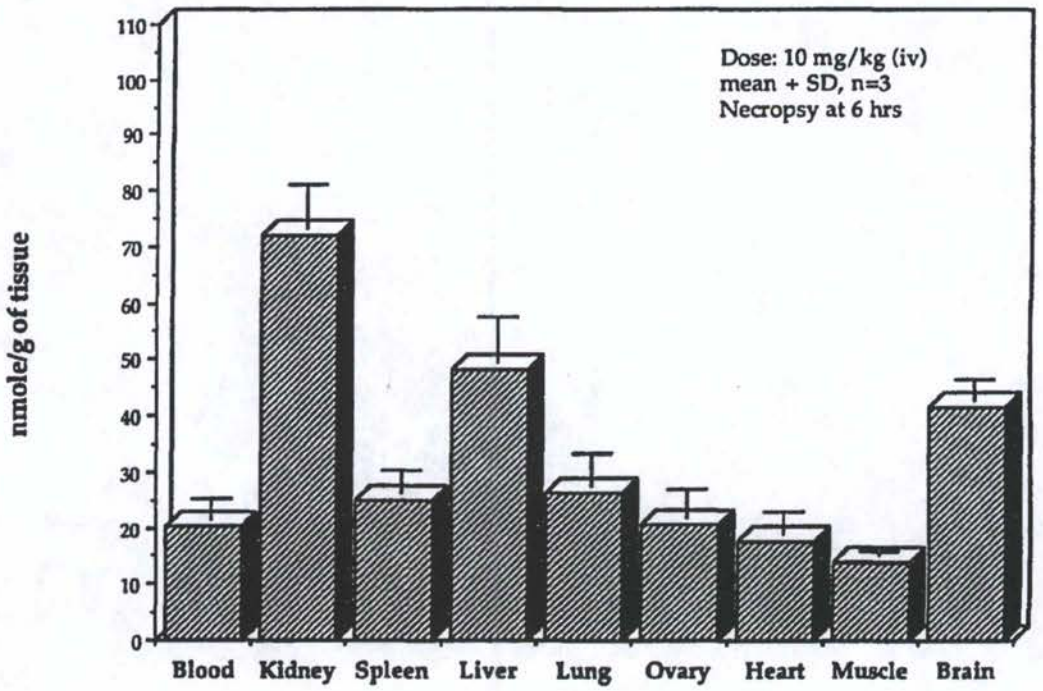

Figure 3.5 Tissue distribution of radiolabel at 6 hr following an intravenous dose of ^{14}C-VCD (10 mg/kg; 10 μCi/rat). Data are expressed as the mean of nmol/g of tissue ± S.D., (n=4).

HPLC Radiochromatogram of Bile from Female F-344 Rats Dosed with ^{14}C-VCD

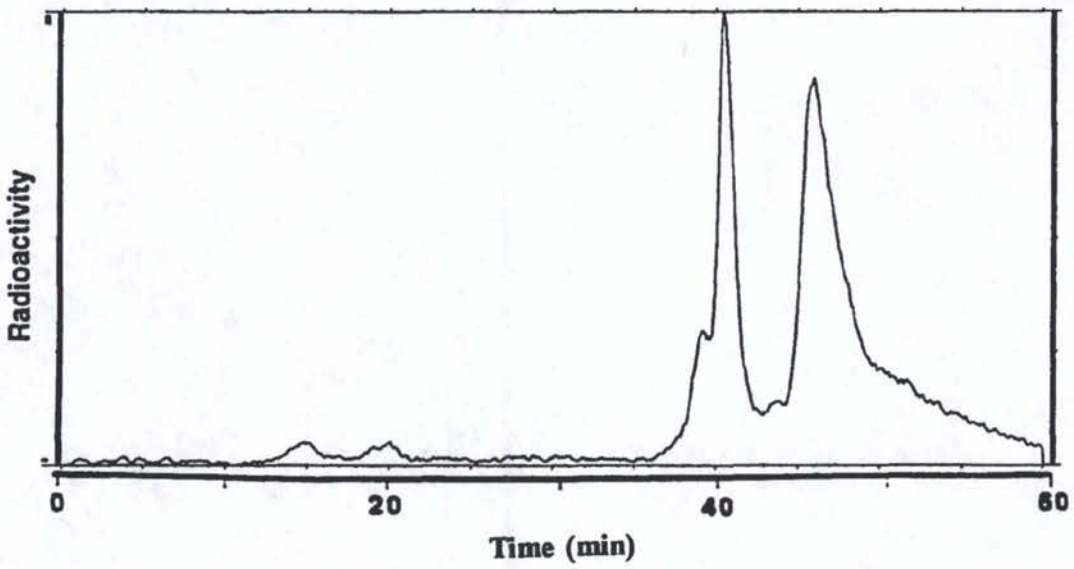

Figure 3.6 HPLC radiochromatogram of bile from female Fischer 344 rat following intravenous administration of ^{14}C-VCD (10 mg/kg; 10 μCi). Bile samples were collected at 30 min intervals for 6 hr. HPLC conditions are described in methods.

Radiochromatograms of Bile from Female F-344 Rats Pretreated with BSO and Synthetic GSH-^{14}C-VCD Conjugate

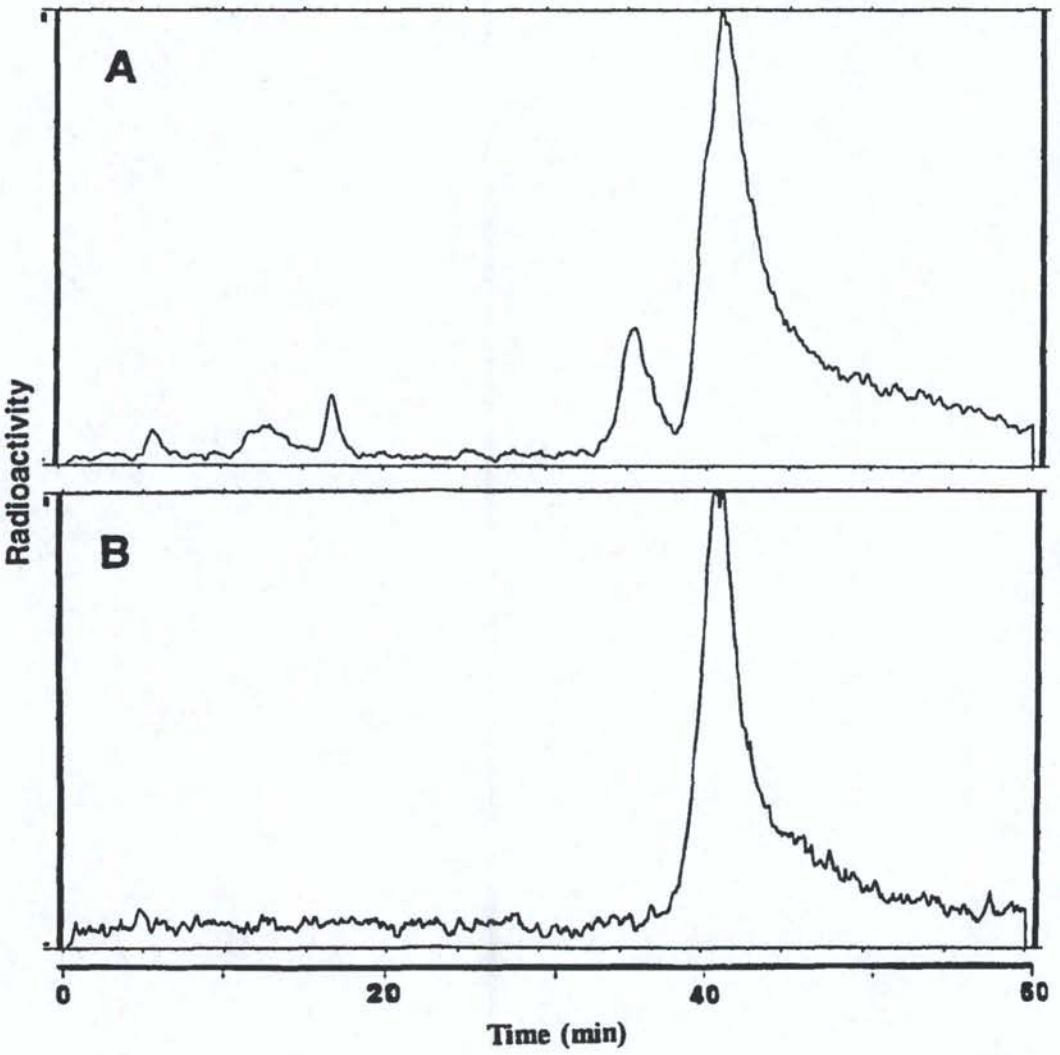

Figure 3.7 Radiochromatograms of: A) bile from female Fischer 344 rats (pretreated with 2 g/kg BSO) following an intravenous dose of ^{14}C-VCD (10 mg/kg; 10 μCi), and B) synthetic GSH-^{14}C-VCD conjugate. Chromatography conditions were identical for both radiochromatograms and are described in methods.

^{14}C-VCD conjugate (Figure 3.7). This indicates that at least one of the polar metabolites present in bile from rats dosed with ^{14}C-VCD may be a GSH conjugate. HPLC analysis of urine samples indicated the tetrol as the major metabolite accounting for approximately 70% of the radiolabel (Figure 3.8). Also, present in the urine were two very polar metabolites which may also be conjugates of VCD. These two metabolites were a less polar than the biliary metabolites.

HPLC Radiochromatogram of Urine from Female F-344 Rat Administered ^{14}C-VCD (pretreated with BSO)

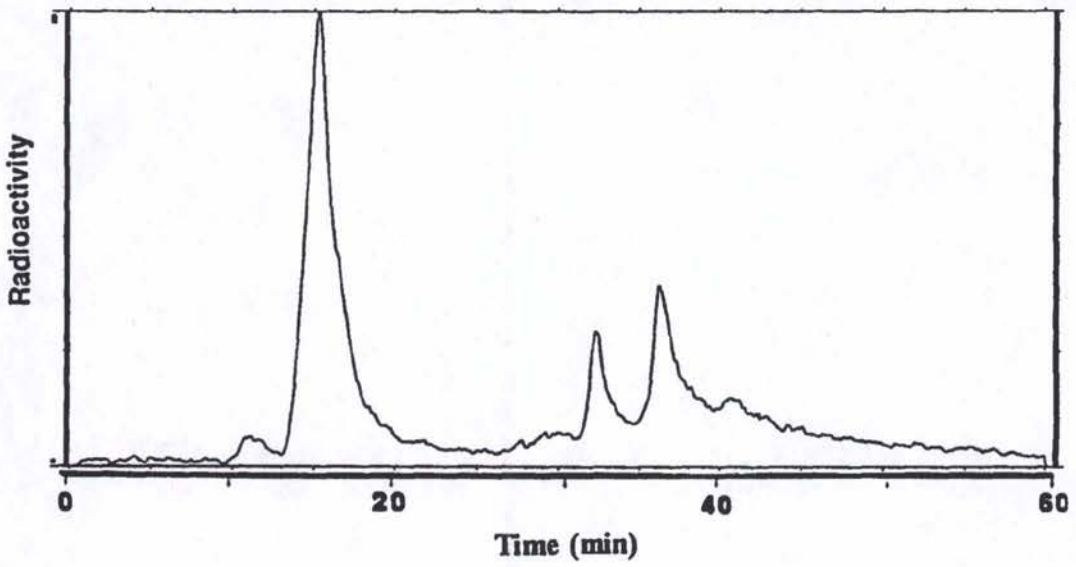

Figure 3.8 Radiochromatograms of urine from female Fischer 344 rats following an intravenous dose of ^{14}C-VCD (10 mg/kg; 10 µCi). Rats were pretreated with 2 g/kg BSO (i.p.). Urine was pooled over a 6 hr period after administration of dose. Chromatography conditions are described in methods.

DISCUSSION

Female $B6C3F_1$ mice are more susceptible to VCD-induced ovarian toxicity as compared to female Fischer 344 rats (NTP, 1989; Chhabra et al., 1990a and 1990b; Smith et al., 1990b). In the preceeding chapter a species difference was observed in the disposition and metabolism of VCD between the species following intraperitoneal administration. Results indicate the rat eliminates VCD more rapidly than the mouse. Also, a difference in the urinary profile suggests different pathways of metabolism of VCD between the species. These differences in the disposition and metabolism may in part explain why the mouse is more susceptible to VCD-induced toxicity. To characterize the species difference further, the toxicokinetic parameters of a single intravenous dose of ^{14}C-VCD was determined in female Fischer 344 rats and $B6C3F_1$ mice. Also, the biliary excretion of VCD and its metabolites was investigated in female Fischer 344 rats.

Following an intravenous dose, VCD was rapidly cleared from the plasma in both rats and mice. The plasma concentration-time profile of VCD indicates the elimination of ^{14}C-VCD from plasma was biphasic which is consistent with a two compartment model (Figure 3.1 and 3.2). The faster plasma $t_{1/2}$ and shorter MRT of the mouse indicates this species clears VCD from the plasma more rapidly than the rat. This is also supported by the greater systemic clearance (C_L). In contrast, the tetrol metabolite had a much longer MRT value in both species. This metabolite also displayed a much greater AUC value when compared to the parent compound (Table 3.1). The toxicokinetic data suggest that VCD is rapidly biotransformed to the tetrol metabolite and the latter had a more prolonged plasma concentration-time profile compared to the parent VCD in both species.

The volume of distribution, or the apparent body space into which VCD can distribute, was five fold greater in the mouse compared to the rat. VCD distributes from the plasma to tissues, possibly target tissues (ie. ovaries), more profoundly in the mice than in the rats. These results collaborate earlier findings where Sipes et al., (1989) investigated the disposition of a single dermal application of ^{14}C-VCD in female Fischer 344 rats and B6C3F$_1$. They found the tissue:blood ratio of radiolabel in mouse ovarian tissue was five fold greater compared to rat ovarian tissue. Also, results from the preceeding chapter are consistent with the ovarian tissue:blood ratio which was at least two fold higher in the mouse compared to the rat. The species difference observed in ovarian tissue:blood ratio may in part explain why the mouse is more susceptible to VCD-induced ovarian toxicity.

Identification of the only plasma metabolite of VCD in both species, tetrol, was accomplished by cochromatography with authentic standard. The authentic standard was identified by LC-MS analysis and is described in chapter two (Figure 2.8). In the disposition study described in the preceeding chapter, the tetrol was also identified as a urinary metabolite. These results suggest VCD is metabolized to the tetrol in both species, present in the blood, and is eliminated by the kidneys without being further metabolized.

Biliary excretion is an important contributing source to fecal elimination of xenobiotics and their metabolites; therefore, the biliary excretion of VCD was determined. The results indicate that VCD was rapidly metabolized and 17% of the dose was excreted in the bile in female Fischer 344 rats. In the preceeding chapter which described the disposition of VCD, fecal elimination of radiolabel accounted for approximately 5% of the dose. The difference in biliary and fecal excretion suggests that enterohepatic recirculation of VCD and/or its metabolites is occurring. Depletion of

hepatic GSH by BSO pretreatment significantly reduced the biliary excretion of radiolabel to 4.5% of the administered dose (Figure 3.7). A previous study by Giannarini et al. (1981) reported a single, large dose of VCD (500 mg/kg; i.p.) depleted 96% of mouse liver GSH. The dose of VCD used in these studies also depleted hepatic and ovarian GSH (Chapter 4). Taken together, these studies suggest the involvement of hepatic GSH in the metabolism of VCD and its biliary excretion.

Bile flow can directly affect the rate of biliary excretion of compounds. Results from the biliary excretion studies indicate that a single bolus dose of VCD (10 mg/kg; i.v.) does not alter the bile flow rate, nor did pretreatment with BSO. These results indicate the difference in biliary excretion of radiolabel in control and GSH depleted rats is not due to a decrease in bile flow.

HPLC analysis of bile indicates less than 1% of the radiolabel present in the bile was parent compound (Figure 3.6). The tetrol, formed by the hydration of both epoxides of VCD, was a minor biliary metabolite. The majority of radiolabel excreted in the bile was present in at least two very polar metabolites, possible conjugates of VCD. These probable conjugates were not hydrolysed by either ß-glucuronidase or sulfatase treatment, which suggest they are not glucuronide or sulfate conjugates. However, the most polar of these probable conjugates coeluted on HPLC with a synthetic GSH-^{14}C-VCD conjugate (Figure 3.7). These results indicate that metabolism of VCD, either hydration of its epoxides or GSH conjugation, is a prerequisite for biliary excretion. In general, low molecular weight compounds, such as VCD and the tetrol, are poorly excreted in the bile. However, in the rat, compounds with molecular weights greater than 325 (Klaassen and Rozman, 1991), such as GSH-VCD conjugates, can be excreted in appreciable quantities.

GSH has many cellular functions, one of these is to participate in the detoxification of many foreign and endogenous substances (Meister and Anderson, 1983). In this detoxification role, GSH acts as a nucleophilic reagent which reacts with electrophilic centers of xenobiotics or their metabolites. VCD has two epoxides which represent electrophilic centers; thus, these epoxides are capable of reacting with a strong nucleophile such as GSH. Therefore, theoretically VCD can form a mono-GSH conjugate with either of its epoxides or it can form a di-GSH conjugate where both epoxides are conjugated with GSH. If GSH forms a mono GSH-VCD conjugate, the remaining epoxide can still react with cellular macromolecules, such as nucleoproteins and DNA.

In summary, this study was undertaken to determine if a species difference exists in the toxicokinetic parameters of VCD between female Fischer 344 rats and B6C3F$_1$ mice. The results indicate that VCD was rapidly metabolized to tetrol and eliminated from the plasma in both species. However, the volume of distribution of VCD was five fold greater in the mouse compared to the rat. This indicates that VCD distributes from the plasma to tissues, possibly the ovaries, more profoundly in the mice than in the rats. The difference in the volume of distribution may in part explain the greater susceptibility of B6C3F$_1$ mice to VCD-induced ovarian toxicity.

CHAPTER 4

IN VITRO METABOLISM OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE AND ITS MODULATION OF GLUTATHIONE LEVELS IN FEMALE FISCHER 344 RATS AND B6C3F$_1$ MICE

INTRODUCTION

In chapter two the disposition and metabolism of a single intraperitoneal injection of ^{14}C-VCD was compared in female Fischer 344 rats and B6C3F$_1$ mice. The majority of the dose was excreted in the urine by both species. However, the urinary metabolite profile was different between the two species. In rat, the tetrol was the major urinary metabolite, with small amounts of other polar meabolites. In contrast, the tetrol was only a minor urinary metabolite in B6C3F$_1$ mice, and the major metabolites appeared to be polar conjugates of VCD. Also, in chapter 3 the toxicokinetic parameters following a single intravenous dose of ^{14}C-VCD were determined in female rats and mice. Tetrol was the only plasma metabolite identified in both species. Results from these *in vivo* studies indicate both species hydrolyze the epoxides of VCD, however the rat appears to excrete more of this metabolite compared to the mouse. This difference in excretion probably reflects differences in the activities of epoxide hydrolase between the species. A species difference in the kinetic constants of epoxide hydrolase toward VCD may explain in part the species difference in susceptibility to VCD-induced ovarian toxicity.

Conjugation with glutathione (GSH) is another pathway for metabolism of VCD as suggested in chapter three. Epoxides and arene oxides are generally good substrates for GSH conjugation. Hayakawa et al. (1975) examined the ability of VCD, among 50 different epoxide and arene oxides, to form GSH conjugates in sheep liver cytosol. GSH conjugation of VCD was not detected under these experimental conditions. However, styrene oxide, cyclohexene oxide (Hayakawa et al. 1975), and 3,4-epoxyvinylcyclohexane (Boyland and Williams, 1965), which are structurally similar to VCD, were reported to be good substrates for GSH.

Although, Hayakawa et al. (1975) reported that VCD was not a substrate for glutathione-S-transferase, in another study, Giannarini et al. (1981) reported that in male, Swiss albino mice, VCD depleted liver GSH by 96% within 2 hr of administration. The depletion of liver GSH may be due to formation of GSH conjugates with VCD and/or its metabolites (Giannarini et al., 1981) when VCD was administered.

A dramatic reduction in the biliary excretion of ^{14}C-VCD equivalence was observed in rats pretreated with BSO (to deplete hepatic GSH). These results and the fact that the biliary metabolites of VCD are not hydrolyzed by ß-glucuronidase and sulfatase suggest a key role for GSH in the disposition of VCD. VCD most likely conjugates with GSH as suggested by the coelution of a VCD biliary metabolite with a synthetic GSH-^{14}C-VCD conjugate.

Because of the important role of epoxide hydrolase and GSH in the disposition of VCD, the following studies were undertaken to examine species difference in microsomal epoxide hydrolase activity towards VCD and in the ability of VCD to affect the levels of GSH in the plasma, liver, and ovaries of both species. Because of a reduced ability of the mouse to convert VCD to the tetrol, more VCD may be available in the mouse to deplete GSH. A difference in enzyme activity or availability of cosubstrate necessary for detoxification of VCD may contribute to VCD-induced ovarian toxicity.

MATERIALS AND METHODS

Animals

Female B6C3F$_1$ mice and female Fischer 344 rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) at 21 days of age. Rats were housed in hanging wire cages and mice in polycarbonate plastic pans with hardwood chip bedding. The animals

were allowed free access to food (Teklad 4% mouse/rat; Harlan Teklad, Madison, WI) and water. The animals were maintained on a 12 hr light/dark cycle, ambient room temperture of 21-22°C. The animals were acclimated for at least 7 days prior to use.

Chemicals

4-vinylcyclohexene diepoxide, 2-vinylpyridine, and 5,5'-dithiobis[2-nitrobenzoic acid] were purchased from Aldrich Chemical Co. (Milwaukee, WI). Radiolabelled ^{14}C-4-vinylcyclohexene diepoxide (sp. act. 6.0 mCi/mmol) was obtained from Chemsyn Science Laboratories (Lexena, KS). 5-Sulfosalicylic acid, oxidized glutathione, reduced glutathione, glutathione reductase type IV (from Bakers yeast), and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). CarboSorb^{R} and FLO SCINT™ II cocktail were obtained from Packard Instruments (Chicago, IL). Universol™ Cocktail was purchased from ICN Radiochemicals (Irvine, CA). All other chemicals and chromatography solvents used were either analytical grade or HPLC grade.

Chromatography

^{14}C-VCD purification procedure and HPLC conditions are as described in chapter two. VCD and its metabolites were separated and quantified from reaction mixtures using the same HPLC conditions as previously described in chapter two. Gas chromatography conditions and procedure used to assess purity of unlabeled and radiolabeled VCD are also described in chapter two.

Microsomal Preparation

Female Fischer 344 rats and B6C3F$_1$ mice were euthanised by carbon dioxide inhalation. Livers were quickly removed and homogenized in 4 volumes of cold (4°C)

phosphate buffered saline (pH=7.4) with a Potter-Elvehjem Thompson tissue grinder. Liver microsomes were prepared by differential centrifugation as previously described (Hodgson and Dauterman, 1980). Microsomes were stored in 20% glycerol (v/v) at -70 °C for up to 3 months. Microsomal protein content was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Microsomal Incubations

Preliminary studies defined optimal assay conditions for linear metabolite production with substrate, protein concentration, incubation time, and pH. The standard incubation mixture contained 50 mM Tris buffer (pH=9.0), ^{14}C-VCD (5-500 μM) and liver microsomal protein (0.5-1.0 mg/ml) in a total volume of 0.5 ml. Incubations were performed in a shaking water bath maintained at 37 °C. Reactions were initiated by the addition of microsomes. To destroy enzymatic activites for control reactions, microsomes were denatured by submersion of tubes in boiling water for 15 min. At the end of incubation (0-15 min), reactions were terminated by addition of ice cold methanol (0.5 ml), vortexed and centrifuged to precipitate protein. Samples were stored at -70 °C and analyzed within 4 days.

Effect of VCD on GSH and GSSG Levels

Female B6C3F$_1$ mice and Fischer 344 rats (28-30 days old) were given a single intraperitoneal injection of VCD (80 mg/kg) dissolved in Mazola corn oil. Control animals received Mazola corn oil (0.1 ml). The dose was selected based on the ovarian toxicity studies of Smith et al. (1990b). Since GSH levels follow circadian rhythm, VCD was always administered at 10:00 A.M. Following the dose, animals were killed by

carbon dioxide inhalation at 0, 1, 2, 6, 12, and 24 hr, and blood, liver and ovaries were removed immediately.

Determination of GSH and GSSG Content

To determine GSH and GSSG levels, plasma and liver were prepared as described by Anderson (1985) and analyzed individually. To determine ovarian GSH/GSSG concentration, ovaries from each animal were pooled, weighed, and homogenized, with 5% 5-sulfosalicylic acid, in 5 vol/g of wet tissue weight, using a 1 ml Wheaton tissue grinder. Aliquots of the ovarian homogenate were assayed for GSH and GSSG content similar to plasma and liver..

GSH and GSSG were measured using the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and GSSG reductase recycling procedure. This assay is based on the following reactions:

$$1)\ 2GSH + DTNB \longrightarrow GSSG + TNB$$

$$2)\ GSSG + NADPH + H^{+} \xrightarrow[\text{Reductase}]{\text{Glutathione}} 2GSH + NADP^{+}$$

GSH is oxidized by 5,5'-dithiobis (2-nitrobenzoic acid) to yield GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB) (equation 1). The rate of TNB formation is followed at 412 nm and is proportional to the sum of GSH present. To quantify GSSG, reduced GSH present in the sample is derivatized by treatment with 2-vinylpyridine. Following the derivatization, GSSG is reduced to GSH by the action of the highly specific GSSG reductase and NADPH. The reduced GSH is then quantitated using DTNB (equation 1).

RESULTS

In Vitro Studies on the Epoxide Hydrolase Hydrolysis of VCD

Studies were performed to define optimal assay conditions such that metabolite production was linear with substrate, protein concentration, time, and pH. The formation of tetrol was linear over a protein concentration range of 0.25 to 1.0 mg/ml and 75 μM VCD (Figure 4.1). However, product formation was not linear at 15 and 150 μM substrate concentrations at protein concentrations of 0.25 and 1.0 mg/ml, respectively. As shown in figure 4.2 maximum product formation occurred at a buffer pH of 9.0 for incubation mixtures. Tetrol formation was linear over 15 min incubation period for both rat and mouse microsomal reactions. Optimal incubation conditions were different for microsomes prepared from female Fischer 344 rats and $B6C3F_1$ mice. Conditions for mouse microsomal incubations required twice the protein (1.0 mg/ml) and incubation period (10 min) to quantify product formation at low substrate concentrations.

In Vitro Microsomal Metabolism of ^{14}C-VCD

VCD metabolism to the tetrol was investigated in liver microsomes prepared from female Fischer 344 rats and $B6C3F_1$ mice. Spontaneous hydrolysis of the epoxides of ^{14}C-VCD was less than 0.5% at 5 μM and did not exceed 2% at 500 μM. The spontaneous hydrolysis at each substrate concentration, derived from control reactions with heat denatured microsomes, was subtracted from corresponding enzyme-mediated reactions. The data are presented as the Michaelis-Menton plot in Figure 4.3 and 4.4. The apparent kinetic parameters for microsomal epoxide hydrolase activity were derived from Lineweaver-Burk plots (Figure 4.5 and 4.6) and are shown in Table 4.1. The K_m

Effect of Rat Liver Microsomal Protein Concentration on VCD Metabolism

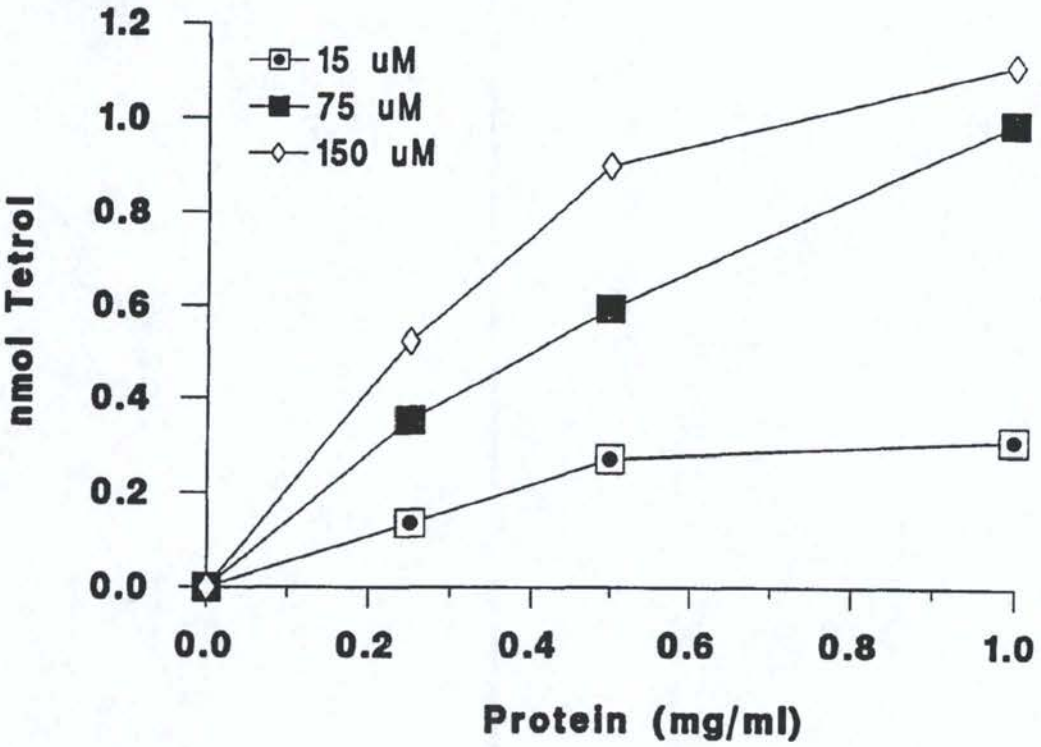

Figure 4.1 Effect of microsomal protein concentration toward the metabolism of three different concentrations of VCD. Buffer pH was 9.0 and incubation period was 5 min for all reactions. Other reaction conditions are described in methods. Data represent the mean of duplicate incubations.

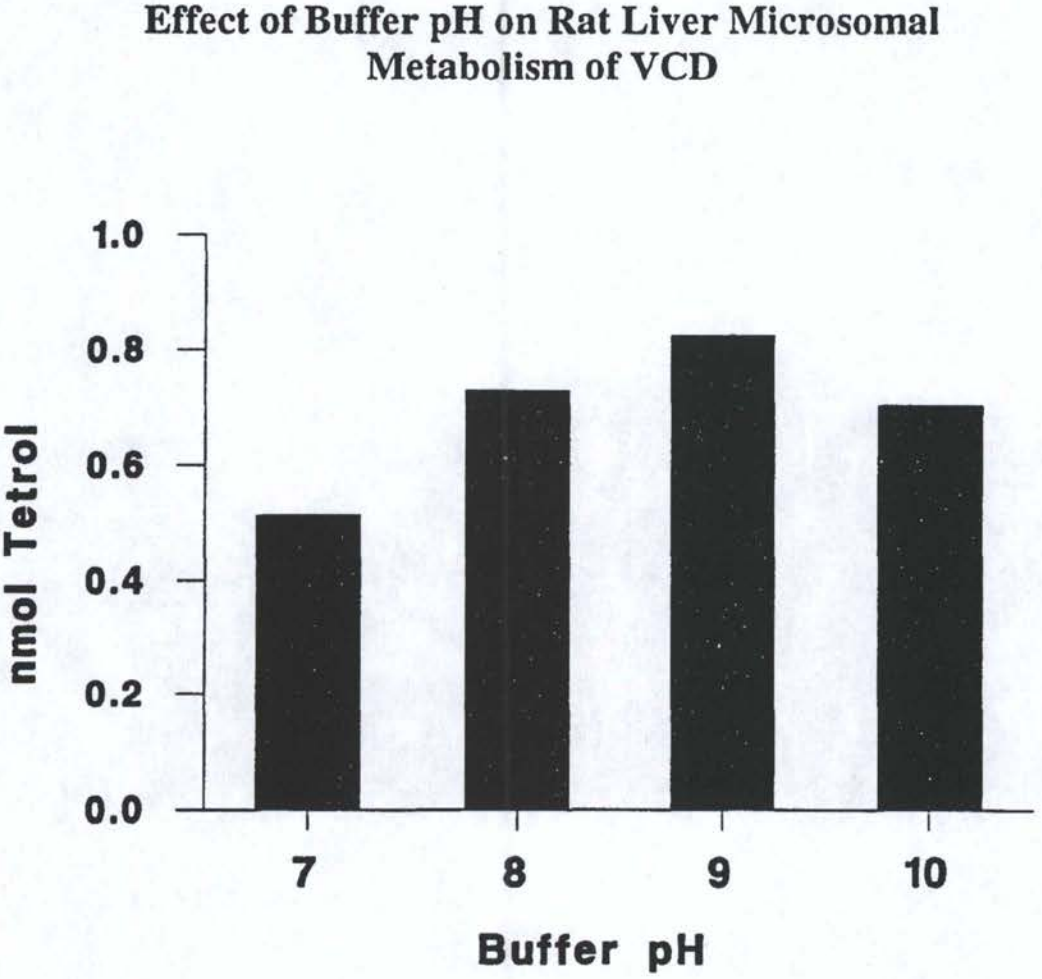

Figure 4.2 Effect of buffer pH on rat liver microsomal metabolism of VCD. Substrate (100 μM) and protein concentration (0.5 mg/ml) and incubation period (5 min) were all held constant. Other reaction conditions are described in methods. Data represent the mean of duplicate incubations.

Female B6C3F$_1$ Mouse Liver Microsomal Metabolism of VCD

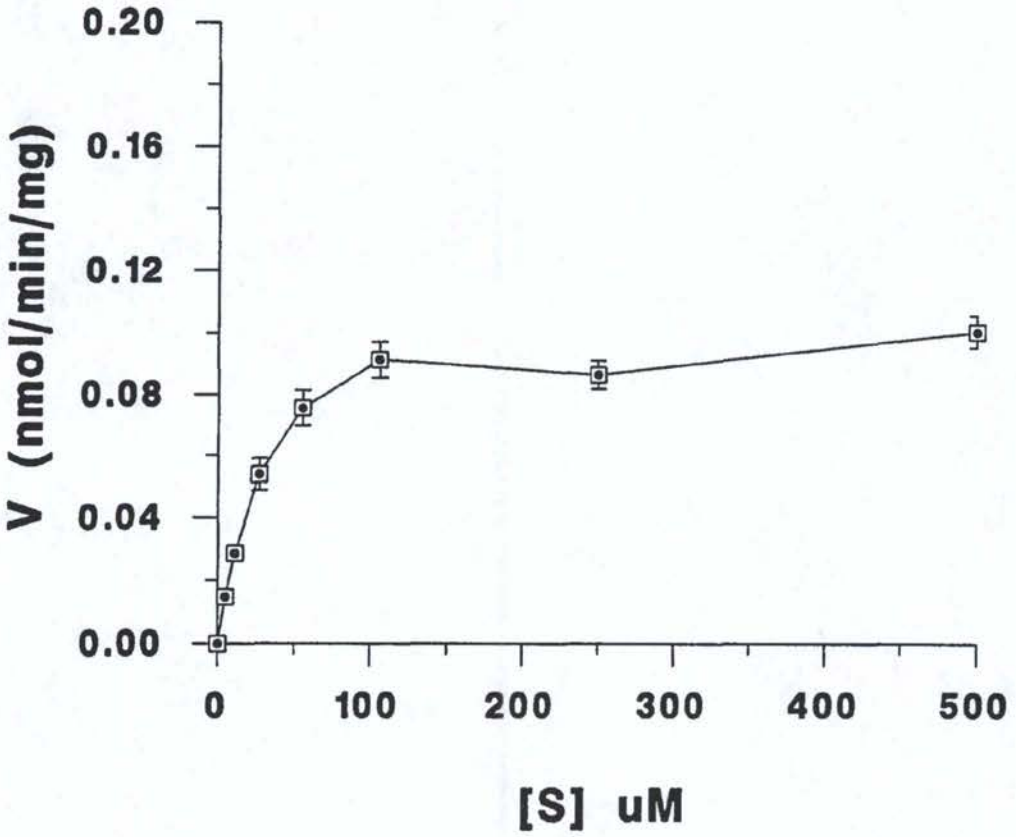

Figure 4.3 Michaelis-Menton plot of the velocity of mouse liver microsomal conversion of VCD to the tetrol metabolite. ^{14}C-VCD (5-500 μM) was incubated with 0.5 mg/ml mouse liver microsomes for 10 min. Other reaction conditions are described in methods. Data represent the mean ± S.D. (n=3-4).

Female Fischer 344 Rat Liver Microsomal Metabolism of VCD

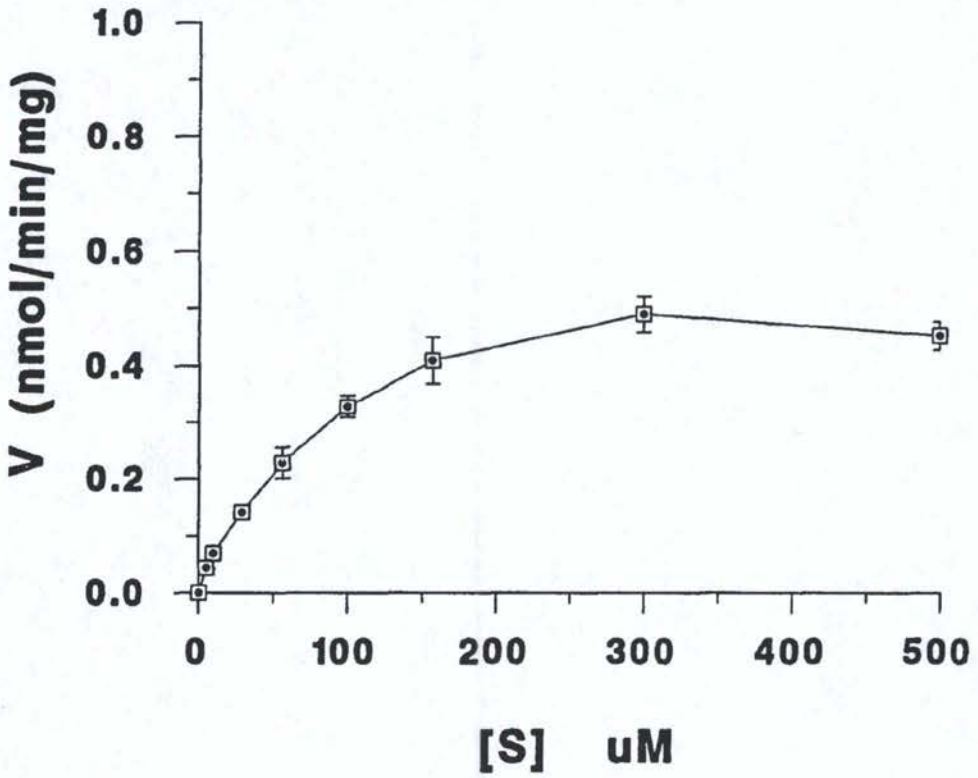

Figure 4.4 Michaelis-Menton plot of the velocity of rat liver microsomal conversion of VCD to the tetrol metabolite. ^{14}C-VCD (5-500 µM) was incubated with 0.5 mg/ml rat liver microsomes for 5 min. Other reaction conditions are described in methods. Data represent the mean ± S.D. (n=3-5).

Lineweaver-Burk Analysis of Female B6C3F$_1$ Mouse Liver Microsomal Metabolism of VCD

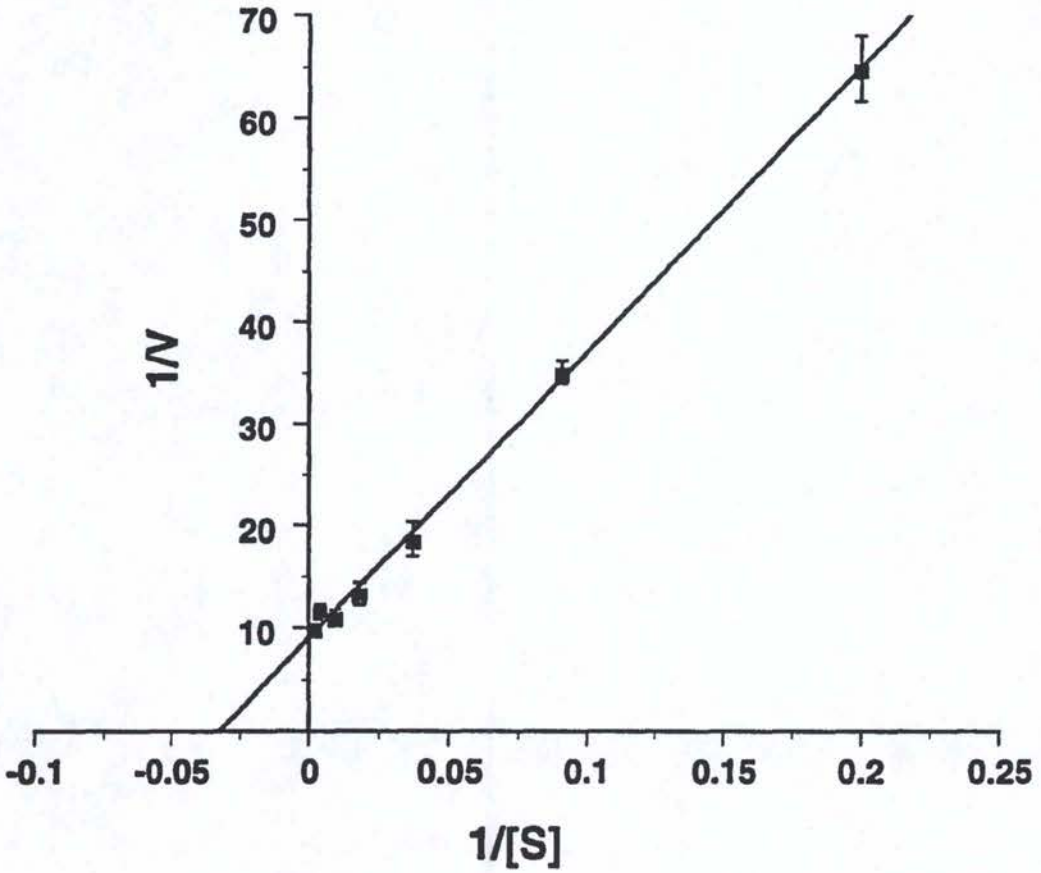

Figure 4.5 Lineweaver-Burk (double reciprocal) plot of mouse liver microsomal epoxide hydrolase activity toward the conversion of VCD to the tetrol metabolite. Reaction conditions were 0.5 mg/ml microsomal protein, buffer pH=9.0, and 10 min incubation period. Other reaction conditions are described in methods. Data represent the mean ± S.D. (n=3-4).

Lineweaver-Burk Analysis of Female Fischer 344 Rat Liver Microsomal Metabolism of VCD

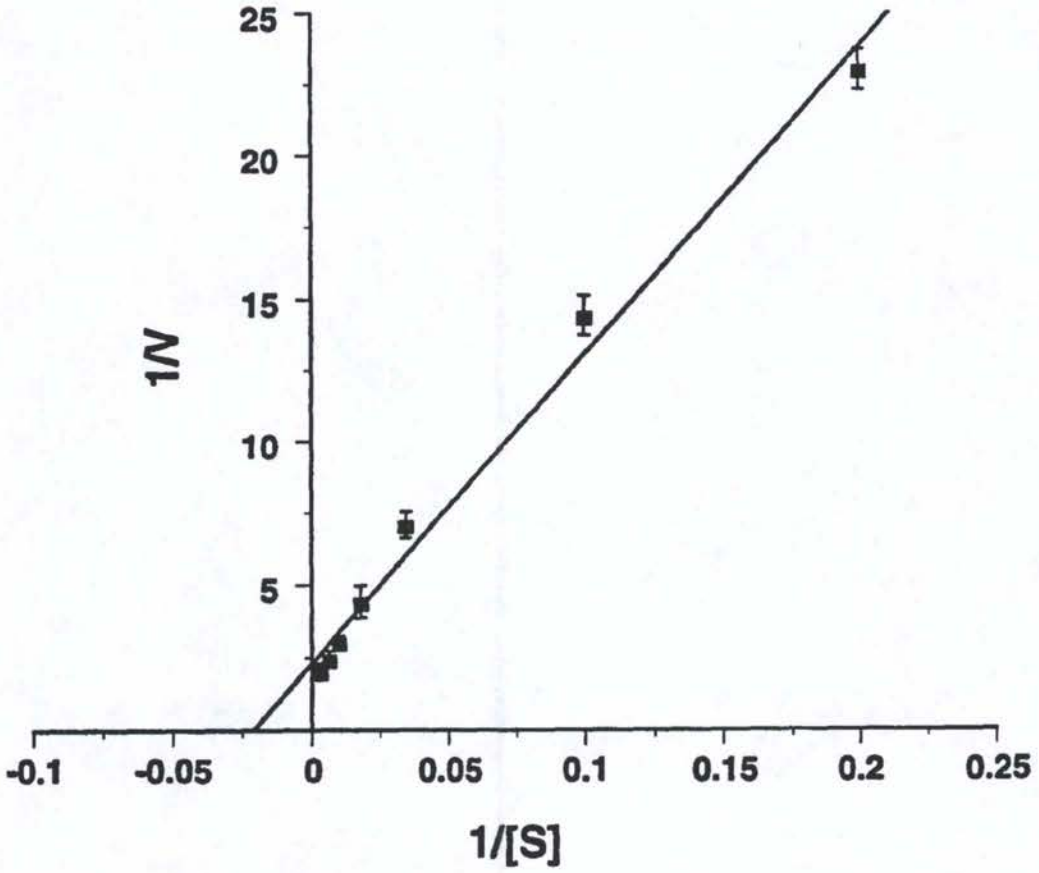

Figure 4.6 Lineweaver-Burk (double reciprocal) plot of rat liver microsomal epoxide hydrolase activity toward the conversion of VCD to the tetrol metabolite. Reaction conditions were 0.5 mg/ml microsomal protein, buffer pH=9.0, and 5 min incubation period. Other reaction conditions are described in methods. Data represent the mean ± S.D. (n=3-5).

Kinetic Parameters of Liver Microsomal Epoxide Hydrolase Conversion of VCD to the Tetrol Metabolite

	K_m (μM)	V_{max} (nmol/min/mg)
Fischer 344 Rat	46.8	0.435
B6C3F_1 Mouse	30.8	0.110

Table 4.1 Apparent kinetic parameters of microsomal epoxide hydrolase activity toward the conversion of VCD to the tetrol metabolite. Liver microsomes were prepared from female Fischer 344 rat and B6C3F_1 mice. Values were derived from Lineweaver-Burk (double reciprocal) analysis. Incubation conditions are described in methods.

values were similar in both species. However, the V_{max}, the maximal velocity, was four times more rapid in the rat compared to the mouse.

Effect of VCD on GSH and GSSG Levels

GSH and GSSG levels were determined in plasma, liver, and ovaries of female Fischer 344 rats and $B6C3F_1$ mice following a single dose of VCD (80 mg/kg; i.p.). VCD did not alter GSSG levels in any tissue examined in the rat or mouse over a 24 hr period (data not shown).

Two hr following VCD administration, hepatic GSH levels were decreased 30% in the rat and 65% in the mouse (Figure 4.7). At 12 hr, hepatic GSH levels returned to control values in the rat, but in the mouse levels remained decreased by almost 30%. Hepatic GSH levels in the mouse returned to control levels 24 hr following VCD administration. Rat ovarian GSH levels were decreased by 25% at 2 hr following the dose of VCD, but returned to control levels by 12 hr (Figure 4.8). Mouse ovarian GSH levels were decreased by 55% at 2 hr and were still decreased by 20% at 12 hr after VCD treatment. The plasma GSH levels in both species were significantly decreased at 2 hr, but returned to control levels in both species at 12 hr following the dose.

Hepatic Glutathione Levels in Female Fischer 344 Rats and B6C3F$_1$ Mice Following VCD Administration

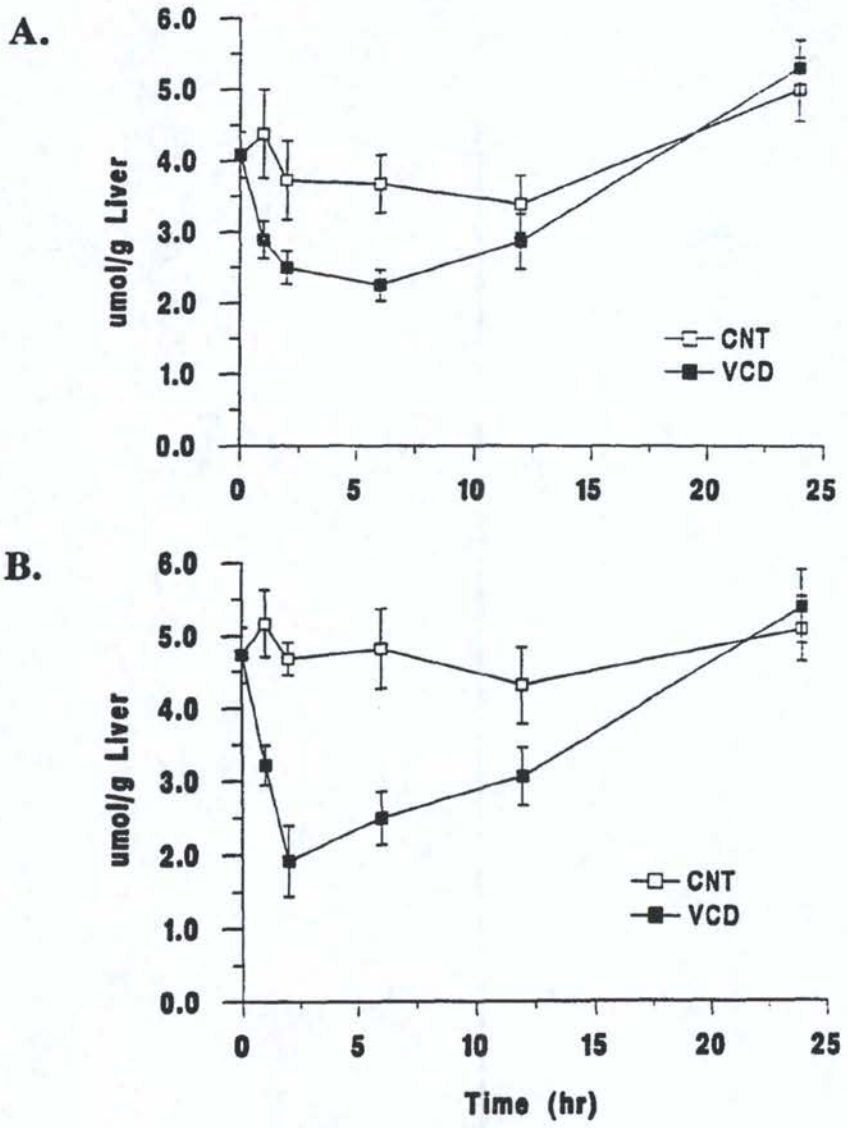

Figure 4.7 A) Female Fischer 344 rats and B) B6C3F$_1$ mice were administered a single dose of VCD (80 mg/kg; i.p.) at 10:00 A.M. Animals were killed at selected times over 24 hr, blood removed, and plasma analyzed for GSH content as described in methods. Data represent the mean ± S.D. (n=3-5).

Ovarian Glutathione Levels in Female Fischer 344 Rats and B6C3F$_1$ Mice Following VCD Administration

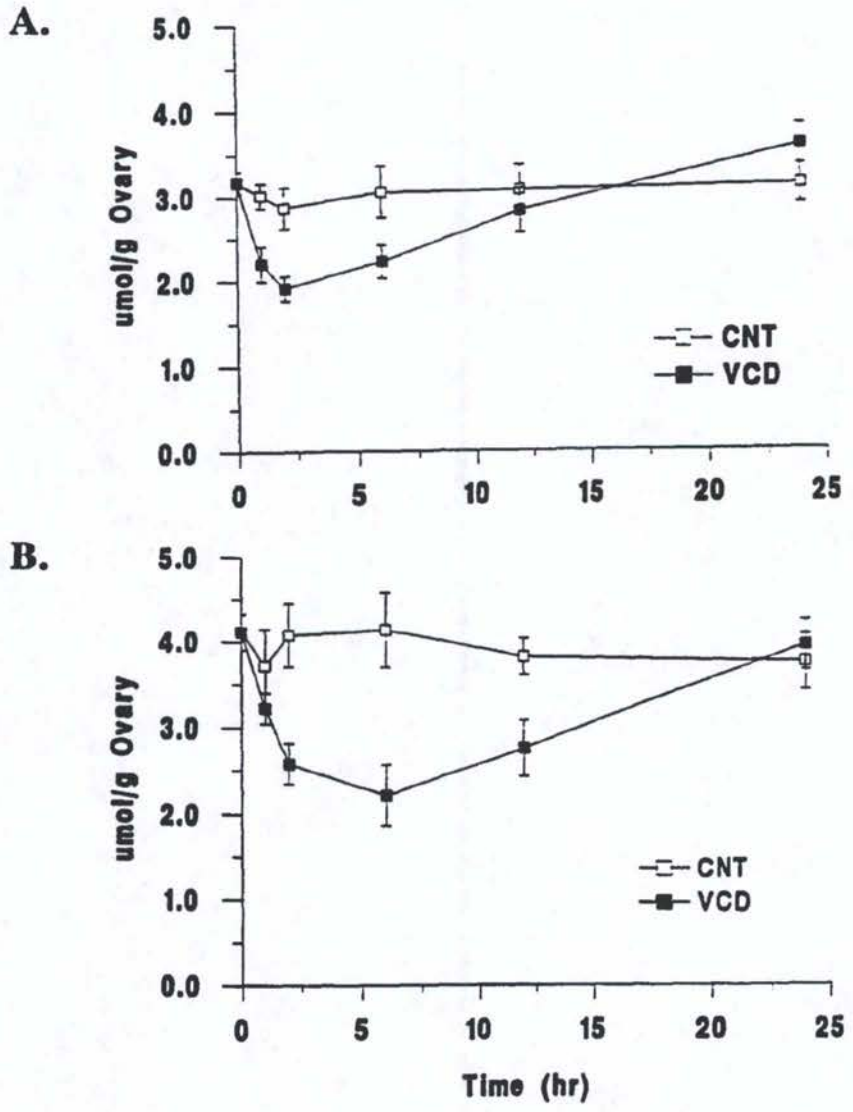

Figure 4.9 A) Female Fischer 344 rats and B) B6C3F$_1$ mice were administered a single dose of VCD (80 mg/kg; i.p.) at 10:00 A.M. Animals were killed at selected times over 24 hr, ovaries removed, and analyzed for GSH content as described in methods. Data represent the mean ± S.D. (n=3-5).

Plasma Glutathione Levels in Female Fischer 344 Rats and B6C3F$_1$ Mice Following VCD Administration

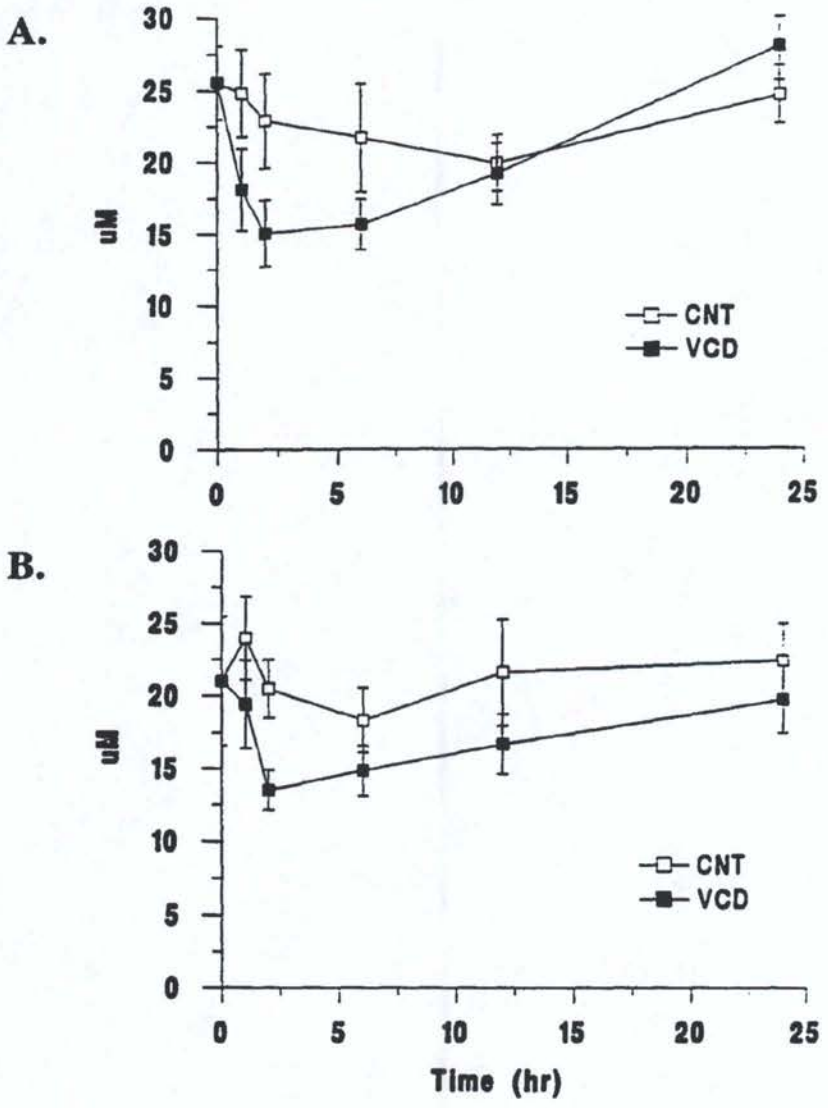

Figure 4.8 A) Female Fischer 344 rats and B) B6C3F$_1$ mice were administered a single dose of VCD (80 mg/kg; i.p.) at 10:00 A.M. Animals were killed at selected times over 24 hr, livers removed and analyzed for GSH content as described in methods. Data represent the mean ± S.D. (n=3-5).

DISCUSSION

The rationale for this study was based on previous results which have shown that female B6C3F$_1$ mice are more susceptible to VCD-induced ovarian toxicity when compared to Fischer 344 rats (NTP, 1989; Chhabra et al., 1990a & 1990b; and Smith et al., 1990b). To investigate this species difference, chapter two examined the disposition of a single dose of ^{14}C-VCD in both species. Results indicate that the rat eliminates radiolabel more rapidly than the mouse. Also, a difference in the urinary metabolite profile suggests different pathways of metabolism of VCD between the species. The major difference in the urinary excretion profile is the rat excretes the tetrol as the major metabolite, whereas the mouse primarily excretes conjugates of VCD. These studies provided the rationale for directly studying the metabolism of VCD by hepatic microsomal epoxide hydrolase of both species.

The objective of this study was to determine the kinetic constants for the epoxide hydrolase catalyzed conversion of VCD to the tetrol. Liver microsomes from female Fischer 344 rats and B6C3F$_1$ mice were one source of epoxide hydrolase. The results indicate the V_{max}, or the maximum rate, was four-fold more rapid in rat liver microsomes compared to mouse liver microsomes. The K_m, or the affinity of the enzyme for the substrate, was similar in both species. Recently, Keller (1993) reported the V_{max} for the microsomal hydolysis of VCD, to be nine-fold higher in the rat than in the mouse liver. Keller also found the K_m for rat liver microsomal epoxide hydrolase to be six-fold higher than mouse liver microsomes. One reason for the differences in kinetic constants may be that Keller (1993) used hepatic microsomes from Crl:CD^{R}BR rats instead of Fischer 344 rats. Another reason for the differences may be the procedure used to assess metabolism. Keller measured the rate of disappearance of VCD instead of

the rate of formation of the product of metabolism. These reasons may account for the differences observed in the kinetic constants between the studies.

Another objective of this study was to determine if a single dose of VCD depletes GSH, or causes an increase in GSSG levels, to a greater extent in one species compared to the other. The maximum decrease of hepatic GSH was 30 and 65% in the rat and mouse, respectively. GSH levels returned to control values at 12 hr in the rat and 24 hr in the mouse. A previous study by Giannarini et al. (1981) reported a single dose of VCD depleted 96% of mouse hepatic GSH. The GSH concentration levels returned to control values 24 hr following the dose. This discrepancy in depletion of liver GSH levels is most likely due to the much larger dose of VCD (500 mg/kg; i.p.) used by Giannarini et al. (1981) as compared to the dose of 80 mg/kg (i.p.) used in the present study.. The dose used in the present study was selected because it was shown to be very effective at causing ovarian toxicity in both the Fischer 344 rat and $B6C3F_1$ mouse when administered daily for 30 days (Smith et al., 1990b).

Previous studies have shown that VCD caused a dose and time dependent loss of small oocytes in the ovaries of $B6C3F_1$ mice and Fischer 344 rats (Smith et al., 1990b; Chhabra et al., 1990b). Therefore, ovarian GSH and GSSG levels were determined in both species following a single dose of VCD. At 2 hr, ovarian GSH levels were decreased by 25% in the rat and 50% in the mouse. However, at 12 hr GSH levels returned to control values in rat ovaries, but were still decreased by 20% in mice ovaries. Ovarian GSH levels for both species returned to control levels at 24 hr.

A single dose of VCD did not alter GSSG levels in any tissue examined in either species. This indicates that a single dose of VCD does not cause oxidative stress in these two species. Based on the greater reduction of GSH and no increase in GSSG levels, it appears that mice conjugate VCD with GSH more readily than rats. These findings correlate well with previous disposition studies where the major urinary metabolites in mice were very polar conjugates, whereas, in rat urine, the major metabolite was the tetrol of VCD.

In summary, this study explored species differences in microsomal epoxide hydrolase activity toward VCD, and in VCD-induced depletion of hepatic and ovarian GSH. The results indicate the V_{max}, or the maximum rate for the conversion of VCD to the tetrol was four-fold more rapid in rat liver microsomes compared to mouse liver microsomes. The difference in velocities correlate with results from the disposition study where the tetrol was the major metabolite present in rat urine, whereas, in the mouse it was only a minor metabolite. The effect of VCD on the concentration of hepatic and ovarian GSH correlates with the difference in profile of urinary metabolites. The reduced capacity of mouse microsomal epoxide hydrolase to convert VCD to the tetrol, results in exposure of the mouse ovary to higher levels of VCD. This explains the greater VCD-induced reduction observed in mouse ovarian GSH. The results may also explain, in part, why $B6C3F_1$ mice are more susceptible to VCD-induced ovarian toxicity than Fischer 344 rats. By causing a greater reduction in ovarian GSH, the mouse ovary may be more susceptible to the potentially tissue damaging effects of VCD.

CONCLUSIONS

The objective of this dissertation was to further elucidate the basis for the species difference observed between Fisher 344 rats and B6C3F$_1$ mice to VCH-induced ovotoxicity. Previous work in our laboratory demonstrated that differences in bioactivation of VCH to reactive epoxides plays a key role in the species difference to the ovotoxic effects of VCH. It was determined that rats bioactivate VCH to a lesser extent, thus follicular depletion does not occur following administration of VCH to rats. However, following administration of the epoxide metabolites of VCH, VCM and VCD, ovarian toxicity was observed in rats. Because the epoxide metabolites were less potent in mice than rats, it was suggested that differences in detoxification of these reactive metabolites may also play an important role in the species difference observed between mice and rats. Therefore, the purpose of these studies was to determine if differences in the two major detoxification pathways of VCD, namely epoxide hydrolase and glutathione conjugation, exist. Studies were aimed at examining the diepoxide of VCH because it is the most potent metabolite in terms of follicular depletion, and because it represents an occupational hazard.

The most significant observation regarding the species difference in the metabolism of VCD was that the mouse had a reduced capacity to detoxify VCD when compared to the rat. It appears that the rat utilizes two enzyme pathways to metabolize VCD, hydration of the epoxides by epoxide hydrolase and conjugation of the epoxides by glutathione S-transferase. The mouse, however, primarily utilizes the latter pathway. The results of several studies support this conclusion. First, results from the disposition study indicate the excretion rate of VCD metabolites was more rapid in the rat as compared to the mouse. The slower excretion rate of VCD metabolites suggests that the

mouse ovary may be exposed for a longer duration to VCD. Second, the urinary metabolites were different between the two species. In the rat, the tetrol was the major urinary metabolite. Also present as minor metabolites were polar conjugates of VCD. In contrast to the rat, the major metabolites excreted in mouse urine were the polar conjugates, and tetrol was present as a minor metabolite. Third, the V_{max} for the *in vitro* conversion of VCD to the tetrol metabolite was four-fold more rapid in rat liver microsomes as compared to mouse liver microsomes. This difference in V_{max} correlates with results from the disposition study. Here it was found that the tetrol was the major metabolite present in rat urine, whereas, in the mouse it was only a minor metabolite. Fourth, VCD caused a greater and more prolonged decrease in the concentration of hepatic and ovarian GSH in mice when compared to rats. The effect of VCD on the concentration of ovarian and hepatic GSH levels corresponds with the differences observed in microsomal epoxide hydrolase activity and in the profile of urinary metabolites i.e. with a reduced capacity to metabolize VCD to the tetrol, more VCD is available to deplete the liver and ovary of GSH. These findings then explain the greater VCD-induced reduction of GSH observed in mouse ovary and liver. Taken together, the results of these studies suggest that one reason the mouse is more vulnerable to VCD-induced ovarian toxicity is because of a reduced capacity to detoxify VCD when compared to the rat.

Recent *in vitro* studies examining the capacity of ovarian rat follicles to detoxify VCD via epoxide hydrolase further support the key role this pathway has in rats. It was shown that small pre-antral follicles, as compared to larger pre-antral follicle, have a reduced capacity to convert VCD to the tetrol by epoxide hydrolase. These *in vitro* studies correlate with previous *in vivo* studies in which VCD has been shown to cause a significant depletion of small pre-antral follicles. Therefore, the susceptibility of these

follicles may be due in part to their reduced capacity to convert VCD to its detoxified metabolite, the tetrol. Although GSH levels were not examined in these follicles, variations in these levels cannot be ruled as another factor involved in the susceptibility of these follicles.

The relationship of the liver and ovary with VCD exposure via the systemic circulation is illustrated in figure 4.10. VCD is distributed to the liver and metabolized by either hydration of the epoxides by epoxide hydrolase and/or conjugation of the epoxides by glutathione S-transferase. Both of these reactions are probable detoxification processess which render VCD more water-soluble and readily excreted. However, if hepatic metabolism enzymes become saturated or lack an essential cosubstrate, as may occur during chronic exposure, the ovary could be exposed to higher levels of VCD via the systemic circulation. The mouse would be more vulnerable to chronic exposure to VCD because of its reduced capacity to hydrolyze the epoxides of VCD. In addition, GSH available for the conjugation of VCD would be more rapidly depleted in the mouse compared to the rat because of the deficit in the epoxide hydrolase activity.

The results from this research indicate that a difference in the disposition and metabolism of VCD exists between female Fischer 344 rat and B6C3F_1 mice. The reduced capacity of mouse microsomal epoxide hydrolase to convert VCD to the tetrol, could expose the mouse ovary to higher levels of VCD. This explains the greater VCD-induced reduction of mouse ovarian GSH. It is concluded that differences in the disposition/metabolism of VCD explain, in part, the increased susceptibility of the mouse ovary to the toxic effects of VCD.

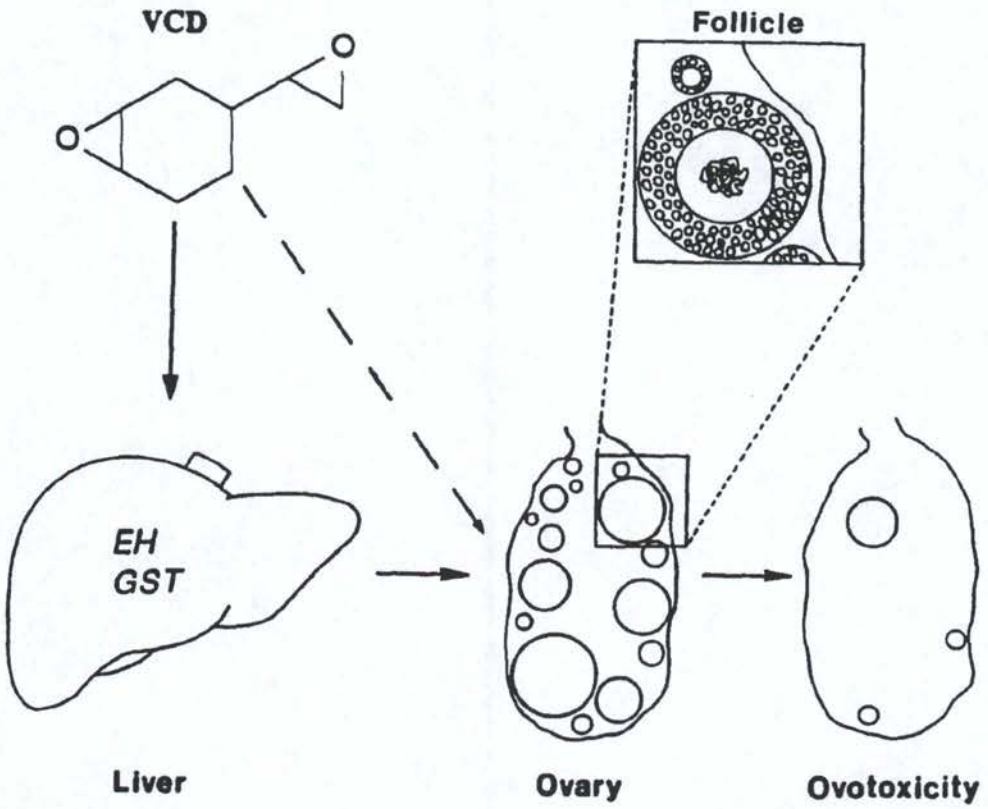

Figure 4.10 The probable relationship of the liver and ovary with VCD exposure. VCD is distributed to the liver and can be metabolized by either epoxide hydrolase (EH) and/or glutathione S-transferase (GST).

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